Elucidation of the Retinoid Signalling Pathway Involved in Axon Guidance in *Lymnaea stagnalis* and *Xenopus laevis*

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Abstract

The vitamin A metabolite, retinoic acid (RA), is known to play a crucial role in several developmental processes including axial patterning and differentiation. More recently, RA has been implicated in the regenerative process acting through its classical signaling pathway, the nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR), to mediate gene transcription. Moreover, RA has been shown to act as a guidance molecule for growth cones of regenerating motorneurons of the pond snail, *Lymnaea stagnalis*. Our lab has recently shown that RA can induce this morphological response independent of nuclear transcription, however, the role of the retinoid receptors in RA-induced chemoattraction is still unknown. Here, I show that the retinoid receptors, RXR and RAR, may mediate the growth cones response to the metabolically active retinoic acid isomers, all-*trans* and 9-*cis* RA, in *Lymnaea stagnalis*. Data presented here show that both an RXR and RAR antagonist can block growth cone turning in response to application of both isomers. Because no prior investigations have shown growth cone turning of individual vertebrate neurons, I aimed to show that both retinoic acid isomers were capable of inducing growth cone turning of embryonic spinal cord neurons in the frog, *Xenopus laevis*. For the first time in *Xenopus*, I showed that both all-*trans* and 9-*cis* RA were able to induce significantly more neurite outgrowth from cultured embryonic spinal cord neurons and induce positive growth cone turning of individual growth cones. In addition, I showed that the presence of the RXR antagonist, HX531, blocked 9-*cis* RA-induced growth cone turning and the RARβ antagonist, LE135, blocked all-*trans* RA-induced growth cone turning in this species. Evidence provided here shows for the first time, conservation of retinoic acid-induced growth cone turning in a vertebrate model system. In addition, these data show that the receptors involved in this morphological response may be the same in vertebrates and invertebrates.

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Table of Contents

List of Figures

Chapter 1: Introduction

1.01 – General Introduction

A regenerative process is often thought of as a recapitulation of the developmental process first utilized to make original connections within the developing embryo. Essentially, during neural development, neuronal somas extend neuritic processes, which are guided through the environment by structures called growth cones. The growth cone acts as a chemosensor that responds to varying gradients of guidance cues to ensure proper target selection. Many guidance cues have been identified, but the cellular pathways by which they mediate their effects have not been fully identified. One such guidance cue, which has been shown to attract and guide neuronal growth cones from the pond snail, *Lymnaea stagnalis*, is the vitamin A metabolite, retinoic acid (RA). Recent work in our lab has investigated the sub-cellular mechanisms involved in this chemoattractive response (Dmetrichuk *et al*. 2006, 2008; Farrar *et al.* 2009). However, due to the complexity of the cellular interactions of RA, there are a number of possible candidates. RA has been classically thought of as a nuclear transcription factor involved in many developmental processes including axial patterning, neuronal differentiation and cell proliferation, working through the nuclear receptors; retinoic acid receptor (RAR) and retinoid X receptor (RXR; Maden, 2007). However, recent evidence suggests a non-classical, non-genomic role for RA in axon guidance through its receptors (Carter *et al.* 2010; Farrar *et al*. 2009).

There is growing evidence for RA acting as a neurotrophic factor and more recently as a guidance cue, which is not uncommon, as many other trophic molecules have been shown to act as guidance cues, primarily in vertebrate model systems. The majority of studies on RA have utilized a vertebrate model, in fact, evidence indicating the potential chemotropic abilities of RA was first shown in vertebrates (Maden *et al.* 1998a). Since then we have extended the studies by looking at the effects of RA on individual growth cones in the pond snail, *Lymnaea stagnalis* and the South-African clawed frog, *Xenopus laevis* as neurons of these species are capable of regeneration *in vitro*.

Therefore, my first aim was to investigate the role of RXR and RAR in RA-mediated growth cone turning in the mollusc, *Lymnaea stagnalis*. I then investigated the conservation of RA's neurotrophic and chemotropic roles in the South-African clawed frog, *Xenopus laevis*. By utilizing embryonic spinal cord neurons from the frog, I determined, for the first time, whether two RA isomers, all-*trans* and 9-*cis*, were able to induce neurite outgrowth from these cultured neurons. I then sought to determine whether these two isomers were able to induce turning in developing growth cones and assess the role of their receptors in mediating this response.

1.02 Axon guidance and the growth cone

Throughout neural development, axons are guided by many different diffusible factors released from both intermediate and final target sites (Ming *et al*., 1997). This process is critical during development for proper initial functional innervation, as well as during regeneration, because innervation of the appropriate target is also required for functional recovery. Currently, it is thought that the main protein families responsible for axonal guidance and repulsion during development and regeneration are ephrins, netrins, semaphorins and slits (Bashaw and Klein, 2010). The latter three have been shown to be involved in commissural axon guidance to ensure proper contralateral and ipsilateral axonal innervation. These proteins are chemotropic factors that are expressed in a gradient in order to exert their specific effects by guiding developing axons.

Regenerating neurons extend processes called neurites and at the tip of every neurite is a structure known as the growth cone (Figure 1A). The growth cone is integral in deciphering the various guidance cues it encounters as it grows. Chemotropic molecules act on the growth cone to either steer the neurite towards (chemoattractant) or away from (chemorepellant) the source of these guidance molecules, whether it be intermediate tissues or terminal target sites. Prior to a growth cone response, internal changes must first occur to enable the structure to turn. Within

the growth cone, local protein synthesis acts upon the internal actin and microtubule cytoskeleton to provide the growth cone and neurite with their overall shape and dynamic movement (Dos Remidios *et al.* 2003). Movement is generated by persistent extension and/or retraction of finger-like projections termed filopodia and the vale-like lamellipodia (Erskine and Herrera, 2007) (Figure 1B). Neurotrophins have been shown to be important regulators of cytoskeletal dynamics responsible for actions such as axonal guidance and may serve this role during neural development (Ming *et al*., 1997). Classically, neurotrophic factors are a group of proteins that are able to promote the survival and growth of neurons. Paves and Saarma (1997) showed that neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were able to direct rat dorsal root ganglion (DRG) growth cone turning (Paves and Saarma, 1997). Many of the guidance cues that the growth cone interacts with lead to activation of calcium (Ca^{2+})-dependent intracellular pathways involving the RhoGTPase family, the most notable of which are Ras homolog (Rho), Ras-related C3 (Rac) and cell division control protein 42 (Cdc42). Their distribution within growth cones result in attraction or repulsion of the growth cone to various chemotropic molecules (Henley and Poo, 2004).

Recent evidence has shown a role for the Vitamin A metabolite, retinoic acid (RA), as both a trophic and tropic factor. Our lab has shown that RA is capable of inducing neurite outgrowth from newt spinal cord explants (Dmetrichuk *et al.* 2005) and cultured snail neurons (Dmetrichuk *et al.* 2006). Furthermore, RA has been shown to act as a guidance cue in the chick (Maden *et al*. 1998a), newt (Dmetrichuk *et al*. 2005) and snail (Dmetrichuk *et al.* 2006; Dmetrichuk *et al*. 2008; Farrar *et al*. 2009).

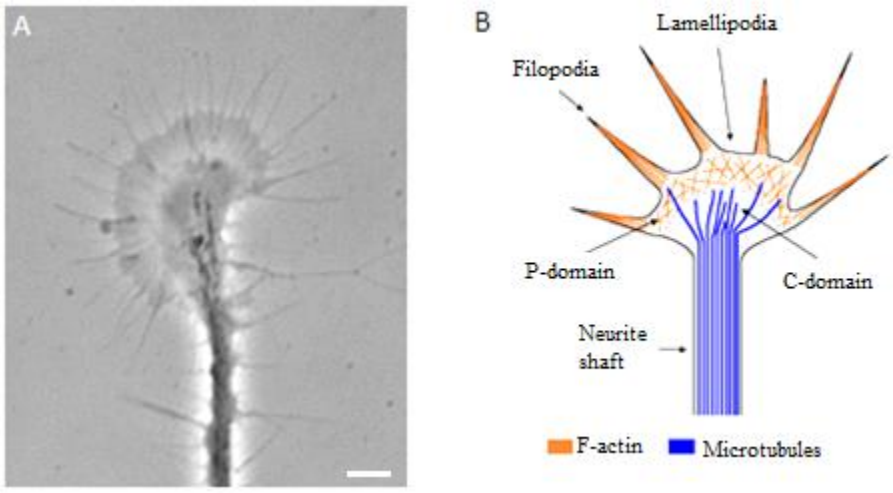

Figure 1. Structure of a regenerating neurite and growth cone. (A) Phase contrast image of a cultured PeA neuron from *Lymnaea stagnalis*. Scale bar = 15µm. (B) A diagram depicting the morphology of and structures comprising the growth cone, most notably the filopodia and lamellipodia. The P-domain and the C-domain represent the peripheral and central domains of the growth cone respectively (Erskine and Herrera, 2007).

1.03 Retinoic Acid

RA is a vital molecule known to play a role in neural differentiation and axial patterning in several different species. In addition, there is growing evidence that RA plays a crucial role in the regenerative process (Carter *et al*. 2010; Carter *et al*. 2011; Corcoran *et al*. 2000, 2002a; Dmetrichuk *et al.* 2005, 2006; Maden *et al*. 1998; Prince and Carlone, 2003). It is unknown whether RA signalling seen in developmental processes is the same, or similar, to that seen in regeneration. However, recent literature has shown that disruption of the RA pathway in regeneration capable animals disrupts their ability to regenerate (Dmetrichuk *et al.* 2005, Carter *et al*., 2011). Many studies have shown that the mammalian CNS retains the capacity to regenerate, but only under specific conditions that provide a permissive environment for neurons to extend processes and form new connections (Chernoff and Stocum, 1995; Ferretti and Whalley, 2008; Silver and Miller, 2004). Interestingly, artificially up-regulating RA levels in animals incapable of regeneration can induce functional recovery in models of spinal cord injury (Agudo *et al*. 2010). In addition, retinoid defects have been shown in a model of motor neuron

disease (Corcoran *et al*. 2002b). Because of the involvement of RA in many developmental and regenerative processes in the nervous system, it is important to understand the mechanism by which it exerts its effects.

i. Retinoic acid synthesis, metabolism and signaling

RA is an endogenous metabolite of Vitamin A that is obtained from the dietary intake of plant carotenoids or animal retinal esters, which can be stored as retinyl esters until needed (Maden, 2007). Vitamin A is metabolically converted to three organic compounds: retinol (alcohol), retinal (aldehyde) or retinoic acid (acid) (Marill *et al*., 2003). Vitamin A circulates within the blood as retinol which is able to bind to retinol-binding proteins (RBPs) until it is required by cells. It can then be shuttled into the cytoplasm of a cell via membrane bound proteins (Figure 2; Niederreither and Dollé, 2008). Once in the cytoplasm, retinol is free to bind cellular retinol binding proteins (CRBP), which can facilitate the oxidation of retinol to retinal via alcohol dehydrogenase (ADH) and to retinoic acid, irreversibly, by the enzyme retinal dehydrogenase (RALDH) (Figure 2; Maden and Hind, 2003; Niederreither and Dollé, 2008). The two most biologically active and well-studied RA isomers are all-*trans* and 9-*cis* retinoic acid (Appendix: Figure 25). It is still unknown as to whether all-*trans* RA and 9-*cis* RA are metabolised through separate mechanisms or through an isomerization reaction from one to the other (Maden and Hind, 2003). Classically, following RA synthesis, retinoic acid can freely bind cellular RA-binding proteins (CRAPBs), which are able to shuttle the molecule into the nucleus for autocrine signalling or out of the cell for paracrine signalling (Figure 2). Once in the nucleus, RA can bind to two nuclear receptor classes, the RARs and the RXRs, which act as transcription factors for various genes. RARs have been shown to bind both all-t*rans* and 9-*cis* retinoic acid and form heterodimers with RXR (Maden, 2007). In contrast, the RXRs were previously thought to bind exclusively to 9-*cis* retinoic acid in vertebrates. However, literature suggests this may not be the case and that all-*trans* RA may also bind to RXRs in vertebrates, albeit at lower affinity than 9-

cis RA (Heyman *et al*. 1992; Ulven *et al.* 2001). In addition, Nowickyj *et al*. (2008) found that both all-*trans* and 9-*cis* RA isomers bind with equal affinity to RXR in the locust. The RXR can form either homodimers, or heterodimers with orphan receptors such as the peroxisome proliferator-activated receptor (PPAR), nerve growth factor-induced clone B (NGFIB) and the nuclear receptor related 1 (NURR-1), which is located in dopaminergic neurons (Mangelsdorf and Evans, 1995; Chambon, 1996; Corcoran *et al*., 2000). The complex formed between the ligand (RA) and receptor (RXR or RAR) then bind to specific DNA sequences located in the promoter region of target genes called retinoic acid response elements (RAREs) and retinoid X response elements (RXREs) (Figure 2; Chambon, 1996; Marill *et al*., 2003). RA receptors are highly conserved between species including humans, rats, mice and the frog, *Xenopus laevis*, all of which have three RARs (α,β,γ) (Blumberg *et al*. 1992; Kastner *et al*., 1994; van der Wees *et al*. 1998) and three RXRs (α,β,γ) (Kliewer *et al*., 1994; Marklew *et al*. 1994). Interestingly, until recently it was thought that only the RXR was present in non-chordate species as no RAR homolog had been identified. However, our lab recently cloned the first non-chordate RAR (*Lym*RAR) in the pond snail, *Lymnaea stagnalis* (Carter, 2011).

In order to maintain homeostasis of RA levels, RA is degraded via the CYP26 family of enzymes into polar metabolites, which can be eliminated from the cell and subsequently from the body. A detailed pathway for RA signalling is shown in Figure 2.

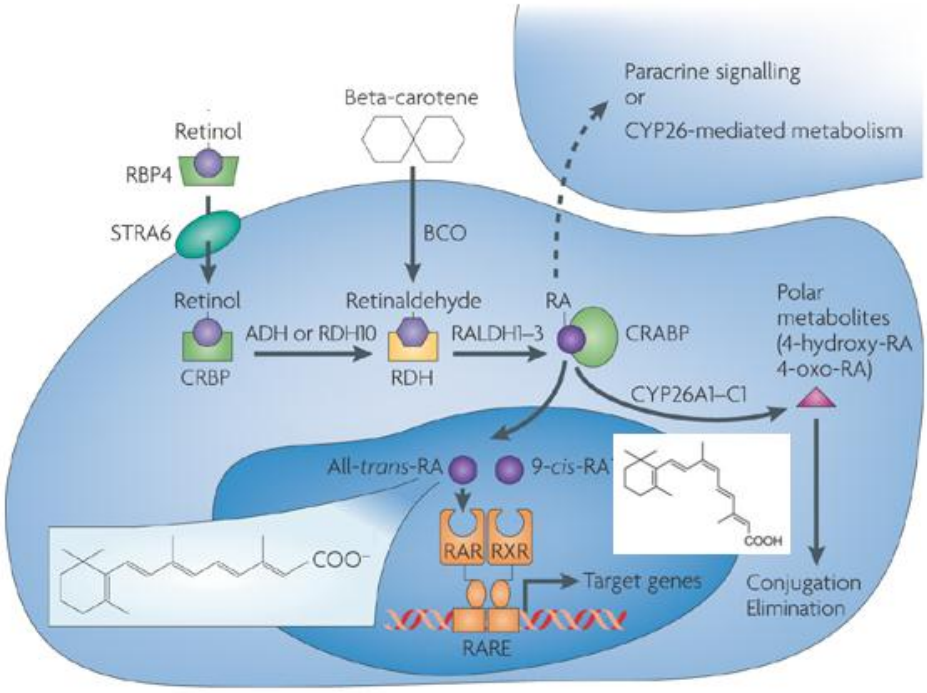

Figure 2. Retinoic acid synthesis and signalling pathway. RBP4: retinol binding protein, STRA6: stimulated by retinoic acid gene 6, CRBP: cellular retinol binding protein, ADH: alcohol dehydrogenase, RDH: retinol dehydrogenase, RALDH: retinal dehydrogenase, CRABP: cellular RA-binding protein, CYP26: cytochrome P450, family 26, RARE: retinoic acid response element. (Adapted from Niederreither and Dollé, 2008).

1.04 Retinoic acid in development and regeneration

RA is a vital biological molecule implicated in several crucial developmental processes including axial patterning, neural differentiation and configuration (Maden and Hind, 2003; Maden 2007). Endogenous RA synthesizing enzymes have been identified in several species at various developmental stages. RALDHs can be detected in the mouse embryo as early as day 7.5 and are expressed following gastrulation (stage 4-5) in the developing chick (Ang and Duester, 1997; Maden *et al*., 1998b). Retinol dehydrogenase (RDH) is localized to the developing CNS, ear and eye as well as the cranial and spinal ganglia, gut and myotomes in the mouse embryo (Romert *et al*., 1998). RA is found in the mouse node and chick Hensen's node (Hogan *et. al.*, 1992; Chen and Solursh, 1992) as well as the *Xenopus* blastopore during gastrulation (Chen *et.*

al., 1994). This evidence shows that RA is indeed synthesized within the embryo and indicates its prevalence throughout development. Furthermore, endogenous RA can be detected in the chick and mouse embryo at varying levels along the neural tube. The forebrain and midbrain contain nearly undetectable levels whereas the region destined to form the spinal cord contains the greatest levels; the hindbrain on the other hand has transitional levels potentially due to diffusion of RA anteriorly, and/or RA degradation by Cyp26 (Maden, 2000). More recently, our lab has shown the presence of the RA receptors, RXR and RAR, in the developing *Lymnaea* embryo and the two biologically active RA isomers in the adult CNS (Carter *et al*. 2010; Carter, 2011; Dmetrichuk *et al*. 2008). Taken together, these data show the presence and metabolism of RA in the embryo required for maintaining normal development.

Due to the widespread production of RA, deficiencies in dietary vitamin A and disruptions in the pathway can cause severe abnormalities. Dickman *et al*. (1997) withdrew vitamin A from the diet of pregnant rats and noted malformation of the face, neural crest, eyes, heart, and nervous system of the developing fetus. Furthermore, White *et al*. (1998) showed a loss of posterior cranial nerves and existence of ectopic otic vesicles in vitamin A deficient (VAD) rats. Investigation into the effects of RAR double mutants revealed that RARαγ null mutants showed severe scoliosis, median facial cleft, axial and limb skeletal malformations, multiple eye abnormalities and glandular abnormalities (ocular and salivary) (Lohnes *et al*., 1994). Moreover, van der Wees *et al*. (1998) expressed a dominant-negative RARβ in *Xenopus* embryos which induced specific hindbrain abnormalities reminiscent of VAD quails that also showed neural tube defects (Maden *et al*., 1996). Furthermore, inhibition of RA synthesis by ethanol application induced severe craniofacial defects associated with fetal alcohol syndrome in the mouse (Deltour *et al*. 1996). Taken together, these examples illustrate the crucial role that RA plays in development, and any interference with the signalling pathway, whether through changes in synthesizing enzymes or the retinoid receptors, results in severe embryological abnormalities.

RA is also implicated in tissue regeneration, which is not unexpected as the processes involved in regeneration of limbs, spinal cord, gut, etc. are ones thought to be involved in embryonic development. Therefore, regeneration can be thought of as a recapitulation of many developmental processes. In addition to the role of RA in development, extensive literature has provided evidence for its role as a trophic factor, more specifically a neurotrophin. Because neurotrophic factors promote the survival and growth of neurons, they are important following injury to minimize damage and to maintain function within the nervous system. It has been suggested that the absence of neurotrophins, (along with inhibiting factors such as glial scars and astrocytic debris), influence the survivability of neurons following physical damage and ultimately lead to cell apoptosis (Chernoff and Stocum, 1995; Ferretti and Whalley, 2008; Silver and Miller, 2004). A number of proteins and molecules have been regarded as neurotrophic factors; the first to be identified, NGF, was isolated and characterized by Cohen and Levi-Montalcini (1956). Since then several other factors have been identified including: BDNF, NT-3 and NT-4/5 in addition to glial cell-line derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), hepatocyte growth factor/scatter factor (HGF/SF) and several members of the fibroblast growth factor (FGF) family (Tonge and Leclere, 2000). Recently, RA has garnered greater interest as a potential neurotrophic agent as many of these properties seen with other trophic factors have been shown with RA.

RA synthesis and signalling have been shown to be up-regulated following injury in several species. For example, Zhelyaznik *et al.* (2003) showed an increase in transcriptional activity of RALDH-2 and cellular retinol-binding protein-1 (CRBP-1) following peripheral nerve crush injury. Furthermore, a focal increase in RALDH-2 activity was seen following spinal cord contusions in rats (Mey *et al.* 2005). This evidence supports the role for RA as a neurotrophin, in that its metabolic up-regulation may serve as a neuroprotectant to shield healthy tissue from damage in the CNS and PNS following injury. Furthermore, inhibition of the RA signalling

pathway resulted in inhibition of tail and spinal cord regeneration in the newt (Carter *et al*., 2011). Moreover, Corcoran *et al*. (2002b) showed that depriving retinoids from the diet caused motorneurons in adult rats to undergo neurodegeneration and that a defect in RARα is found in cases of spontaneous amyotrophic lateral sclerosis (ALS) in rats. In addition, endogenous levels of 9-*cis* RA were found in the limb wound epidermis of regenerating urodeles and limb blastemas of fiddler crabs (Viviano *et al.* 1995; Hopkins, 2001). The evidence presented here points to a role for RA in not only limb and spinal cord regeneration, but suggests that defects in signalling may occur in instances of severe neurodegenerative diseases.

Recently, our lab showed that in the CNS of adult *Lymnaea*, both all-*trans* (atRA) and 9-*cis* RA were present, and found at nearly equal concentrations (Dmetrichuk *et al*. 2008). Furthermore, both of these isomers have been shown to induce significant neurite outgrowth of cultured *Lymnaea* neurons (Dmetrichuk *et al*. 2006, 2008) and newt spinal cord explants (Dmetrichuk *et al*. 2005; Prince and Carlone, 2003; Rand, 2009; Zeglinski, 2008). In addition, several studies have shown atRA induced neurite extension of rat dorsal root ganglion (DRG; Corcoran *et al*. 2000) and embryonic chick neural tissue (Maden *et al*. 1998a). Therefore a physiological role for both these isomers in neural regeneration has been proposed, although the role of 9-*cis* RA in vertebrate regeneration has been less studied. Taken together, these data suggest a role for RA, not only as a neuroprotectant, but also as a trophic factor, where it has the capacity to induce regeneration of vertebrate and invertebrate neural tissue.

1.05 Retinoic acid as a chemotropic molecule

Recently, it has become apparent that RA can act not only as a neurotrophic factor, but also as a tropic factor, where it can guide neuritic processes from regenerating neural tissue. Although indirect, evidence for a chemotropic role for RA was shown in Vitamin A deficient (VAD) quail embryos, where axonal trajectories were found to be disorganized (and extensions reduced) in

the developing neural tube (Maden *et al.* 1996). Furthermore, when a gradient of atRA was applied to dorsal neural tube neurons from the chick, neurites which initially grew evenly (i.e. from all sides) from the soma, encountered this gradient of RA and showed a turning behaviour along the RA gradient (Maden *et al.* 1998a). In contrast, cells that were exposed to atRA in a non-gradient manner did not show a preferential growth trajectory and therefore grew in a dispersed manner. More direct evidence for RA chemoattraction was shown by Dmetrichuk *et al*. (2005) who demonstrated that atRA-soaked beads, which slowly released RA as a localized source, induced directed neurite extensions from adult newt spinal cord explants. Preliminary evidence has shown the same effect with 9-*cis* RA (Rand, 2009; Zeglinski, 2008). In addition, co-culture of newt spinal cord explants with a newt limb blastema induced significant neurite outgrowth on the side of the explant in which the blastema was located (Prince and Carlone, 2003). Interestingly, blocking the RARβ with an antagonist in these co-cultures abolished directed outgrowth. These data suggest that RA may act through the RARβ to influence axon trajectory and target selection in the newt (Dmetrichuk *et al*., 2005).

Although evidence had suggested that RA can direct outgrowth from tissue explants, it had not been shown with individual growth cones. Accordingly, pulsatile micropipette application of atRA to cultured *Lymnaea stagnalis* motorneurons induced positive growth cone turning toward RA, indicating that RA is able to guide individual growth cones and act as a chemotropic molecule (Dmetrichuk *et al.* 2006). More recently, this same phenomenon has been shown with 9-*cis* RA (Dmetrichuk *et al*. 2008; Farrar *et al*. 2009). Classically, RA has been known to work through its nuclear receptors, RAR and RXR, to regulate gene transcription, however, recent evidence has indicated that a novel, non-genomic retinoid pathway may be mediating the growth cone turning response to RA, seen in *Lymnaea stagnalis* cultured neurons (Farrar *et al.* 2009).

1.06 *Lymnaea stagnalis* as a model for the study of RA in regeneration

The pond snail, *Lymnaea stagnalis*, has been utilized in our lab as a model system for studying regeneration and the morphological, molecular and electrophysiological actions of RA (Figure 3A). Unlike vertebrate models, the snail CNS allows us to culture single, identified neurons that have well characterized morphology and function in the CNS (Figure 3B). This procedure ensures that the same cell type is used consistently. In addition, neurons are isolated from adult animals and these cells retain the capacity to regenerate in culture making them great candidates with which to perform various morphological and electrophysiological experiments. In contrast, many vertebrate studies utilize embryonic tissues/cells or PNS neurons/ganglia that retain the ability to regenerate, though, they do not provide the ability to work with single, identifiable adult CNS cells. Furthermore, developmental and regenerational studies can be undertaken in the whole animal (*in vivo*), semi-intact preparation, as well as isolated cell cultures (*in vitro*). In fact, it has been shown that transplantation of single neurons into the brain of intact *Lymnaea* exhibit neurite outgrowth and can restore functional circuitry (Syed et al. 1992). Our lab has shown that by culturing identified cells such as Visceral F (VF) and Pedal A (PeA) neurons, bath application of RA (atRA and 9-*cis* RA) is able to induce a regenerative response by promoting and enhancing neurite outgrowth in the absence of any other neurotrophins (compared to Ethanol treated controls) (Dmetrichuk *et al.* 2006; Dmetrichuk *et al*. 2008). Furthermore, in the isolated CNS preparation, an identified cell demonstrated enhanced neurite extensions in the presence of RA, following a nerve crush injury, which may have been a decrease in degeneration (i.e. neuroprotection) at the site of injury (Vesprini, 2011). In addition to its neurotrophic role, RA has been shown to have a chemotropic role in *Lymnaea* where focal application of RA (atRA and 9-*cis* RA) to an actively growing neurite elicited a positive growth cone turning response towards the source of RA (Dmetrichuk *et al.* 2006; Dmetrichuk *et al.* 2008; Farrar *et al*. 2009). Furthermore, both RA isomers, atRA and 9-*cis* RA, were found to be

present in the CNS and haemolymph of *Lymnaea* indicating that RA likely plays a physiological role in this animal (Dmetrichuk *et al.* 2008).

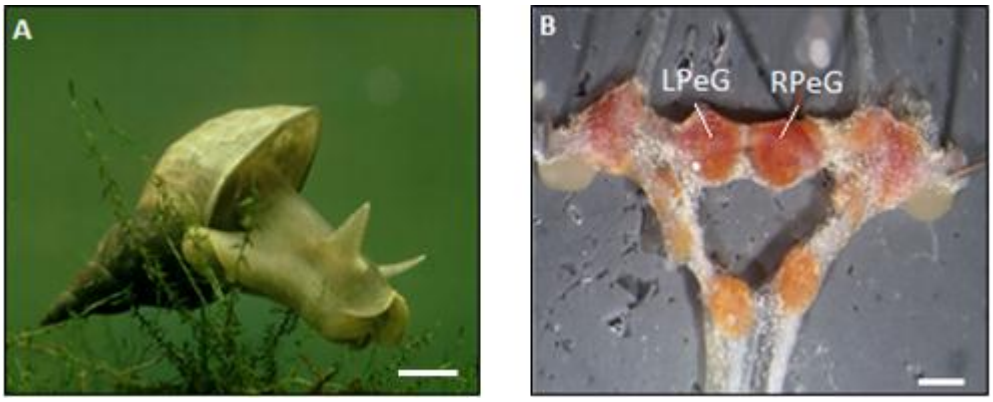

Figure 3. *Lymnaea stagnalis* and the isolated CNS. (A) The mollusc, ***Lymnaea stagnalis*** (Oxford Scientific, 2012; scale bar = 3mm). (B) **The dissected central ring ganglia of *Lymnaea stagnalis.*** LPeG and RPeG: left and right pedal ganglia. Scale bar = 0.25mm (Courtesy of N. Vesprini).

Since RA has been thought to act through its nuclear receptors via the classical pathway, work has been extended to assess the role of nuclear transcription in RA-mediated growth cone turning. First, Farrar *et al.* (2009) showed that bath application of Actinomycin D, an inhibitor of nuclear transcription, did not block RA-mediated growth cone turning, suggesting a possible non-genomic role for RA (contrary to the classical nuclear retinoid pathway). Second, these investigators performed the same growth cone experiments with transected neurites to ensure that the regenerating neurites and growth cones were physically isolated from the soma and therefore prevented any nuclear transcripts from being shuttled to the growth cone over the course of the assay. Interestingly, RA-mediated growth cone turning persisted in the isolated neurites, again suggesting that RA may be working through a non-genomic signaling pathway. Third, in order to determine the necessity of local protein synthesis, anisomycin was applied and RA-induced growth cone turning of isolated neurites was inhibited, suggesting that *de novo* protein synthesis is required to allow growth cone turning in response to RA.

Carter *et al*. (2010) recently cloned and characterized a *Lym*RXR showing presence of the protein with Western blotting in whole snail CNS tissue. Despite being traditionally known as a nuclear receptor, the RXR protein was found in the cytoplasm, neurite and growth cones of cultured neurons using immunohistochemistry. Due to this non-nuclear distribution in neurons, our lab studied a potential non-genomic role of the RXR in the RA-mediated growth cone turning response. Carter *et al.* (2010) found that focal application of the RXR pan-agonist PA024 to isolated neurites induced positive growth cone turning toward the source of the agonist, suggesting that local RXR may mediate this response. Furthermore, bath application of either of the RXR pan-antagonists, PA452 or HX531, inhibited the RXR agonist-induced growth cone turning, suggesting that the actions of the RXR pan-agonist were selectively at the RXR.

It was previously believed that invertebrate species did not possess RARs, however, genomic screening predicted the presence of an ancestral RAR gene in the annelid, *Capitella capitata*, and in the mollusc, *Lottia gigantea*. Based on this evidence, a novel, non-chordate RAR was identified and cloned in the pond snail *Lymnaea stagnalis* (Carter, 2011). Similar to the *Lym*RXR, the *Lym*RAR was present in CNS tissue samples as well as in cultured neurons. Furthermore, the RAR was shown to have a non-nuclear localization in cultured cells whereby staining revealed its presence in the cytoplasm, neurite and growth cones of PeA neurons, a pattern similar to that seen with RXR staining (Carter, 2011). These previous studies, did not however, determine whether this neurite-localized RAR played any role in RA-mediated growth cone turning.

1.07 *Xenopus laevis* as a model

The South-African clawed frog, *Xenopus laevis,* has long been used as a model for the study of developmental processes, as their life stages are well characterized. Many molecular, morphological and electrophysiological techniques have been used with great success in

determining the roles of various molecules and proteins within the adult and embryonic frog nervous system. Furthermore, many investigators have used this model to study regeneration, as the *Xenopus* tadpole retains the ability to regenerate its limbs and tail. One of the main advantages of using this animal is that their eggs are relatively easy to obtain. Gravid females release hundreds of eggs following hormonal injection (or natural processes) and these eggs are robust and easily fertilizable with a high success rate. As stated earlier, staging is well established by Nieuwkoop and Faber (1956) ensuring the experimenter is consistently collecting cells from the proper developmental stage. In our experiments, the stage 22 embryo is used for culturing spinal cord neurons, as this is the stage in which the neural tube is fully formed and a distinct anterior/posterior axis is apparent (Figure 4B). In addition, we are able to assess the effects of a particular molecule, in this case retinoic acid, on a single cultured cell as opposed to other vertebrates such as newts where it is much more difficult. Furthermore, frog embryonic cells are much larger than many other vertebrate cells and these cells have been used extensively for looking at outgrowth and growth cone turning with various guidance cues with great success (Henley and Poo, 2004; Ming *et al*. 1997, 2001; Song *et al*. 1997; Song *et al* 1998; Song and Poo, 1999; Zheng *et al*. 1994). However, unlike the invertebrate model, *Lymnaea stagnalis*, the frog cell cultures consist of a heterogeneous mixture of various cell types, including different neuronal and non-neuronal subtypes. Without using specific cell markers, cells in this case, are identified strictly based on morphology. The neurotrophic and chemotropic effects of retinoic acid on cultured spinal cord neurons have not yet been tested in this amphibian model. Although RA is known to exert neurotrophic effects in vertebrates, its effects in *Xenopus* have not yet been studied. Furthermore, no studies have yet demonstrated that RA can induce single growth cone turning of vertebrate neurons.

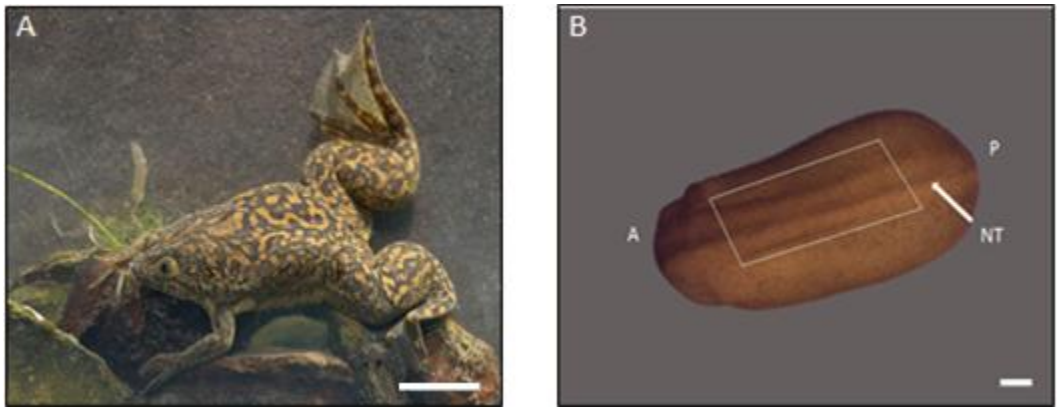

Figure 4. The adult and developing South African clawed frog. (A) The South-African clawed frog, *Xenopus laevis* (van der Voort, 2012, scale bar = 3cm). (B) **The developing embryo of *Xenopus laevis.*** A and P: anterior and posterior ends of the embryo, NT: neural tube. The box depicts the area dissected and used for spinal cord cultures. Scale bar = 0.5mm.

There have been several molecules identified as neurotrophins, originally in an invertebrate model, as potential regeneration-inducing molecules *in vitro*. Many of these molecules were then shown to act as guidance cues in various vertebrate models, including *Xenopus*. The most notable of these are BDNF, Netrin 1, NT-3 and NGF. In addition, other guidance cues have been shown to act on *Xenopus* growth cones including semaphorin-III (Sema III), myelin associated glycoprotein (MAG) and the neurotransmitter Acetylcholine (ACh). It was shown that *Xenopus* spinal cord neurons were attracted to an applied gradient of the neurotransmitter ACh, along with BDNF, Netrin-1, NT-3 and NGF (Zheng *et al*. 1994; Song *et al*. 1997, Ming *et al*. 1997, 2001; Song and Poo, 1999). Conversely, molecules such as Sema-III and MAG act as chemorepulsive agents in *Xenopus* (Song *et al* 1998; Henley and Poo, 2004).

Although the effects these agents have on growth cone guidance is well-established, the pathways they activate were previously unclear. It was shown however, that these guidance cues activated second messenger systems within the neuron and growth cone that ultimately lead to activation or inhibition of the cAMP or cGMP pathways (Song and Poo, 1999). This group showed that the direction of guidance elicited by these cues was dependent on the levels of

cAMP or cGMP within the neurons and growth cone. More specifically, when the cAMP or cGMP pathways were inhibited through their primary mediators, protein kinase A (PKA) or protein kinase G (PKG), the previously chemoattractant guidance cues became chemorepulsive. Interestingly, this process also applied to chemorepulsive guidance cues where activation of these pathways caused chemorepulsive agents to become chemoattractant. Overall, these results suggested that some guidance cues mediate their effects through one of two second messenger systems, either cAMP or cGMP. From this, it was concluded that high levels of either cAMP or cGMP resulted in attraction. Conversely, low levels of each lead to repulsion (Song *et al*. 1998)).

It has been known for decades that Ca^{2+} plays a crucial role in regulating growth cone motility due to its interactions with the cytoskeletal elements within the growth cone. Previous research has shown that *Xenopus* spinal neurons cultured in low Ca^{2+} saline were no longer attracted to the chemoattractant guidance cues such as BDNF and or NGF (Song and Poo, 1999). In addition, large increases in cytosolic Ca^{2+} concentrations have been correlated with growth cone collapse (Bandtlow *et al*. 1993; Gomez *et al.* 1995). In contrast, it has been reported that an increase in $[Ca^{2+}]_i$ in the growth cone of cultured *Xenopus* spinal neurons correlates with an attractive response to a guidance cue (Henley and Poo, 2004). These data suggest that there is an optimum level of Ca^{2+} within the cell and that alterations on either side of this window may induce negative effects. Interestingly, recent evidence from our lab has suggested that acute exposure to high levels of RA elicits a decrease in $[Ca^{2+}]_i$ in the soma of cultured *Lymnaea* Visceral F (VF) neurons, suggesting that the soma may respond differently to neurotrophic and chemotropic agents than do the neurites and growth cones (Vesprini, 2011). However, it is more likely that the differences seen at the growth cone and the soma may be due to the difference in concentrations used in these experiments (higher levels used for soma experiments). Furthermore, previous work by Farrar *et al*. (2009) showed that the calcium channel blocker,

cadmium, blocked RA-mediated growth cone turning, suggesting Ca^{2+} influx is required for this response.

The role of RA in *Xenopus* growth cone guidance and neurite outgrowth has not yet been investigated, however the teratogenic effects of RA during development have been well characterized in this species. As with many other animal models, excess levels of RA during embryogenesis lead to posteriorization of the frog embryo, often resulting in loss of anterior neural tissue and in some cases, embryos lacking heads (Altaba and Jessel, 1991). In addition, RA has been detected at various levels of development, most notably during gastrulation as previously stated. RA is not only present in the embryo but the RAR (α, β, γ) and RXR (α, β, γ) receptors it binds to have been cloned in *Xenopus*, allowing for manipulation of these receptors within the embryo (Sharpe, 1992; Crawford *et al.* 1995; Dollé, 2009). For example, van der Wees *et al*. (1998) expressed a dominant-negative RARβ in *Xenopus* embryos, which induced specific hindbrain abnormalities reminiscent of VAD quails (that also showed neural tube defects) (Maden *et al*., 1996).

Although the role of RA in growth cone guidance in the frog, *Xenopus laevis* has not yet been investigated, RA has been shown to be important in several amphibian models with regards to regeneration. Previous evidence showed that RA was capable of inducing neurite outgrowth in isolated axolotl spinal cords (Hunter *et al*. 1991) and increased mean neurite length and number in the isolated newt spinal cord (Prince and Carlone, 2003; Dmetrichuk *et al*., 2005). Inhibition of retinal dehydrogenase mediated synthesis of RA by disulphiram caused lack of regeneration in the axolotl limb and tail (Maden, 1997). In addition, co-application of atRA and a RARβ antagonist (LE135) to isolated newt spinal cord caused a significant decrease in neurite outgrowth suggesting RA-induced outgrowth is mediated by a member of the RARβ family (Dmetrichuk *et al*., 2005). Furthermore, a significant reduction in neurite outgrowth (~61%) was also seen when explants were co-cultured with primary blastemas in the presence of LE135. This

suggests that atRA is a diffusible factor released from the blastema acting as a neurotrophic factor on cultured spinal cord explants. These features, along with growing evidence for the conservation of RA signalling in development and regeneration in vertebrates, makes *Xenopus laevis* an excellent model in which to conduct growth cone experiments on embryonic spinal cord neurons.

1.08 Objectives

The general objective of this thesis was to elucidate the role of RXR and the recently cloned RAR in RA-mediated growth cone turning in the pond snail, *Lymnaea stagnalis*. Based on prior investigations with an RXR agonist, I hypothesized that RXR mediates the growth cone turning response to RA. However, the role of the novel, non-chordate RAR is still unknown in this species as work with RAR agonists and antagonists have yielded mixed results for both development and electrophysiological effects of RA. Therefore this thesis aimed to determine if there is a role for this novel receptor in RA-mediated growth cone guidance.

Previous literature has indicated that RA has a neurotrophic role in vertebrates, inducing significant neurite outgrowth in several species, (although not shown in *Xenopus*) (Corcoran *et al.* 2002; Dmetrichuk *et al* 2005; Maden, 1998a; Prince and Carlone, 2003). Furthermore, evidence has indicated RA may be able to guide vertebrate neurite outgrowth, but has yet to demonstrate that RA is able to cause turning of individual growth cones, as seen in invertebrate neurons. I thus aimed to show that the two biologically relevant RA isomers, all-*trans* and 9-*cis*, are capable of inducing both a neurotrophic (neurite outgrowth) and chemotropic (growth cone guidance) effect on embryonic spinal cord neurons in the frog, *Xenopus laevis*.

My overall findings support a role for both RXR and the novel non-chordate RAR in RA-mediated chemoattraction in *Lymnaea* neurons. Furthermore, I show a neurotrophic and chemotropic role for RA in *Xenopus* embryonic spinal cord neurons.

Chapter 2: Materials and Methods

2.01 Animals:

All vertebrate animals were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC) and Brock University Animal Aare and Use committee

The pond snail, *Lymnaea stagnalis*, was reared and housed in tanks containing artificial, aerated pond water (Instant Ocean Sea Salt, 5g/L) and was fed daily with romaine lettuce and fish food (Nutrafin Max Spirulina Algae Flake Food). All animals used for cell culture work ranged in size from 12 to 15mm.

The South-African clawed frogs, *Xenopus laevis*, were purchased from Boreal Labs (St. Catharines, ON) and housed in plastic tanks containing chlorine-free water. Females were fed 3-4 pieces of beef liver per day in the week prior to obtaining oocytes, in order to enhance oocyte production. Otherwise, both males and females were fed *Xenopus* pellet food 2-3 times a week.

2.02 Cell-culture procedures:

Lymnaea:

Snails were anaesthetized (25% Listerine in saline) and their central nervous systems (CNSs) were dissected out and given three, five minute antibiotic saline washes (ABS; Gentamicin (225µg/mL) Sigma-Aldrich). The CNSs were treated with trypsin (6mg in 3mL DM (Defined media (Sigma-Aldrich) ranging from 19-20.5 minutes, followed by trypsin inhibitor (6mg in 3mL DM, Sigma-Aldrich) for 10 minutes. Defined media (DM) consists of 50% L-15 (Leibowitz) media, Gentamicin (25ug/mL), 4X salts (NaCl 40mM, KCl 1.7mM, CaCl 4.1mM, MgCl 1.5mM and HEPES 10mM) , Glutamine (60mg/L), D-Glucose (54.05mg/L) and MilliQ water (~24.6%) with a pH of 7.9. The CNS was then pinned out in high osmolarity DM (800µL of 1M Glucose in 30mL DM) followed by removal of the connective tissue sheath covering the Pedal ganglia.

Individual identified motor neurons from the pedal ganglia called Pedal A neurons (PeA; Figure 4) were removed using a fire-polished glass pipette coated with Sigmacote to prevent cell adhesion (approximately 50-60µM in diameter; Sigma-Aldrich). A small amount of suction was applied using a microsyringe to remove individual cell bodies from the CNS. Cells were then placed into poly-L-lysine coated petri dishes (for full procedure refer to Farrar, 2009) containing 2.5mL of conditioned media (CM, see below for details) and 0.5mL of DM. In order to promote neurite outgrowth, all-*trans* retinoic acid was also added (10^{-7}M final concentration) to the culture dishes at the time of cell plating.

Xenopus:

Female frogs were injected with 600 units of Human Chorionic Gonadotropin (Sigma-Aldrich), and then given a 200 unit booster injection the following morning if eggs were not seen. The females were milked to obtain eggs by gently squeezing their abdomen and oocytes were kept in 10% amphibian Ringer's solution (100% Ringer's: 115mM NaCl, 2.6mM KCl, 2mM CaCl2, 10mM HEPES; diluted to 10% using MilliQ water, pH = 7.6). Males were sacrificed by decapitation and after pithing, their testes were removed. Testes were gently disrupted by homogenization and the sperm homogenate was added to the dish of oocytes. Following fertilization, indicated by the dark animal pole rotating upwards in the vitelline envelope in the majority of eggs, residual sperm were removed by washing with 10% Ringer's solution and the zygotes were stored in fresh 10% Ringer's solution at room temperature (RT). All fertilized oocytes were left overnight at RT to develop. Any unfertilized eggs were removed to prevent lysis and contamination of the healthy embryos.

Approximately 24 hours post fertilization, the embryos had reached stage 22 of development (Nieuwkoop and Faber, 1956) characterized by complete formation of the neural tube (Figure 4). The jelly coats and vitelline envelopes were removed with watchmakers forceps

and the fertilized eggs washed in a series of six, five minute washes in 10% Ringer's solution. After the last wash, embryos were transferred to calcium-magnesium free Ringer's solution (CMF-Ringer's; NaCl 115mM, KCl 2.6mM, HEPES 10mM and Ethylenediaminetetraacetic acid [EDTA] 0.4mM; pH = 7.6). The neural tube was then dissected away from the embryo and left in this solution to dissociate the cells and allow for plating. Approximately 30-40 minutes after isolation of the neural tube, dissociated cells were plated using a fire-pulled Pasteur pipette in 3mL of *Xenopus* Defined Medium (*Xen*DM; 50% L-15, 49% Ringer's solution and 1% Fetal Bovine Serum (FBS) pH=7.6; supplemented with Antibiotic and Antimycotic [1mL/100mL]). All-*trans* retinoic acid was added to a final bath concentration of 10^{-7}M in order to promote outgrowth for all growth cone assays. The experiments assessing the neurotrophic effects of retinoic acid had either all-*trans* or 9-*cis* retinoic acid (10^{-7}M) added to the culture dish which was compared to the outgrowth seen in dishes where only vehicle (EtOH; 0.001% in bath) was added. Cells were incubated in each condition for approximately 18 hours before being assessed for outgrowth. Neurites were only measured when the cell could be identified as a neuron based on morphology.

2.03 *Lymnaea* Conditioned Medium (CM) Preparation:

CM was prepared by dissecting 12 *Lymnaea* CNSs from animals ranging in size from 20-30mm. CNSs were then passed through a series of 20 antibiotic saline (ABS, 225µg/ml gentamicin; Sigma-Aldrich) washes, which was repeated prior to each incubation period. The CNSs were then incubated in 7mL of DM at RT and CM produced from incubation periods from day 3-7 or 7-11 were used for cell culture experiments. All CM was checked for contamination prior to use, using an inverted microscope (Zeiss Axiovert 200, 20X and 40X) and discarded if compromised.

2.04 Growth Cone assays:

Lymnaea:

Approximately 18-20 hours following plating of cells, neurons were checked for outgrowth and individual growth cones assessed for active growth prior to the initiation of these experiments. Only growth cones actively growing were used for subsequent assays. A growth cone was monitored for 20 minutes to ensure continued growth and a steady trajectory. All retinoids and chemicals were applied at 10^{-5}M when in the pipette. Test agents included all-*trans* and 9-*cis* retinoic acid, PA024 (RXR agonist), TTNPB (RAR agonist; Tocris Bioscience) and DMSO (vehicle control). These agents were applied to the growth cones using a pressure pipette (Eppendorf-Femtojet). The pipette was placed between 50 and 150 µm from the growth cone depending on both the size of the pipette opening (range = 4-8 µm) and the pressure applied (3-10 hPa). Previous work using pipette assays by Lohof *et al.* (1992) showed that the chemical was at a concentration 100-1000 times less at the growth cone compared to its concentration at the pipette. Therefore the concentration of the agent reaching the growth cone is estimated to be between 10^{-7} and 10^{-8} M. Growth cone turning assays lasted 1 hour and growth cone movement was monitored and photographed throughout (Northern Eclipse imaging software; Empix imaging, ON).

Xenopus:

Cultured spinal neurons were identified based on well-established morphology (Tabti et al. 1998; Undumatla and Szaro, 2001), as the cultures were heterogeneous and contained a variety of neuronal and non-neuronal cell types. In short, cells which displayed a tubular soma and bipolarity were identified as muscle cells and were excluded from these studies. In contrast, cells which displayed a spherical soma with multiple projections (neurites) from the cell body and a similar morphology to a regenerating neuron seen in *Lymnaea* cultures, were identified as

neurons and used for experiments (See Figure 5). All *Xenopus* growth cone turning assays were performed in the same way as previously described for *Lymnaea* growth cones.

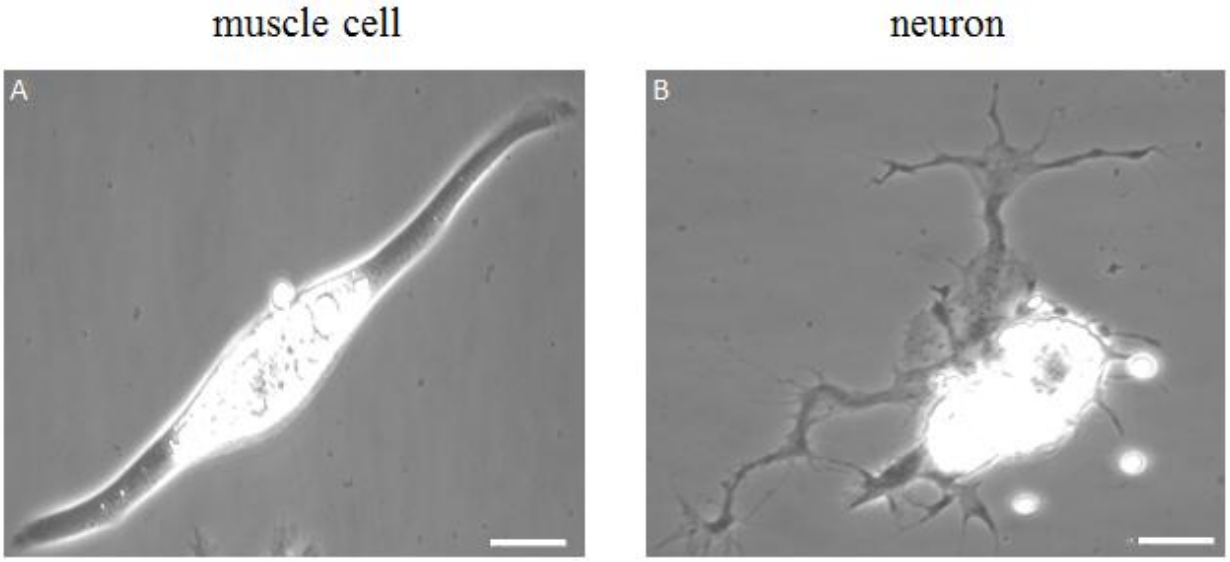

Figure 5. Representative images depicting the morphology of a muscle cell (A) and a neuron (B) in a heterogeneous culture of *Xenopus* neural tube cells. Scale bar = 15 µm

2.05 Chemicals:

Both all-*trans* and 9-*cis* retinoic acid (Sigma-Aldrich) were initially dissolved in 100% EtOH and diluted to a final concentration of 10^{-5}M in DM in a final EtOH concentration of 0.1% which was added to the pipette. Focal application of EtOH alone to growth cones has been shown previously not to elicit positive growth cone turning in *Lymnaea* neurons, so no EtOH controls were repeated in these studies (Dmetrichuk et al. 2006; Dmetrichuk et al. 2008; Farrar et al. 2009). The RXR pan-agonist PA024 and RAR agonist TTNPB were both dissolved in 100% DMSO and diluted to a final concentration of 10^{-5}M in DM and added to the pipette (0.1% DMSO in pipette). In addition, the RXR antagonist HX531 and RAR antagonist LE540 were dissolved in DMSO and diluted to produce a final bath concentration of 10^{-6}M for both *Lymnaea and Xenopus* experiments. DMSO was diluted to a concentration of 0.1% in DM and applied via a pipette (as controls for agonists in the growth cone turning assays). During retinoic acid antagonist experiments, DMSO was added to the dish for a final bath concentration of 0.01% (to

control for vehicle in antagonist experiments). In the *Xenopus* studies, the RARβ antagonist, LE135, was dissolved in DMSO and diluted to a final bath concentration of 10^{-6}M (PA024, HX531, LE540 and LE135 were a kind gift from Dr. H Kagechika. Tokyo).

2.06 Immunostaining:

Lymnaea:

Following the completion of the growth cone assays, cultured neurons were fixed in 4% paraformaldehyde in Phosphate Buffered Saline (PBS) at 4°C overnight. The fixed cells were washed in PBS and then permeabilized in 0.3% Triton X-100 in PBS (PBT) for 20 min and blocked in 5% normal goat serum (NGS) in PBT overnight at RT. The samples were then incubated with the primary anti-*Lym*RXR antibody diluted 1:100 in blocking solution at 4^{o}C overnight. The antibody was designed against a synthetic peptide from the predicted 'hinge' region of the *Lymnaea* RXR, covering the amino acid residues 183-198 between the DNA binding domain (DBD) and ligand binding domain (LBD). This custom made *Lym*RXR antibody was produced in New Zealand white rabbits and affinity purified from the antisera by Pacific Immunology Corp. (Ramone, California, USA). As a control, preparations were also incubated only in blocking solution, without the primary antibody, at 4^{o}C overnight. All samples were then washed 3 times in PBT for 5 minutes and incubated in 1:500 dilution of Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen) in blocking buffer at RT for 2 hours. The samples were washed 3 times in PBT for 5 min and counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 2 min to visualize the nuclei.

Xenopus:

For immunostaining of cultured *Xenopus* spinal neurons, the same protocol employed for *Lymnaea* cells was used. However, to visualize the expression of RARβ2, a *Notopthalmus viridescens* RARβ2-specific antibody diluted 1:100 was used as the primary antibody. The

antibody was designed against a synthetic peptide from the predicted 'hinge' region of the *Nv*RARβ2 covering the amino acid residues 175-188, between the DNA binding domain (DBD) and ligand binding domain (LBD). This custom made polyclonal *Nv*RAR β2 antibody was produced in New Zealand white rabbits and affinity purified from the antisera by Pacific Immunology Corp.

2.07 Analysis:

All images were captured using a Zeiss Axiovert 200 inverted microscope, Q imaging Retiga Exi camera and Northern Eclipse software (Empix Imaging, ON). Individual growth cone turning angles were determined by measuring the angle between the growth cone's initial trajectory and the maximum angle of turning observed over the course of the experiment. Images were taken every 3-4 minutes over the course of each growth cone turning assay. A positive turning response was deemed to have a turning angle towards the pipette and a negative response had an angle away from the pipette. All statistical analyses were performed using SigmaStat software. Data from experiments using 9-*cis* RA were analyzed using a 1-way ANOVA and a Tukey-Kramer *post-hoc* test was used to determine statistical significance. An ANOVA on ranks was performed on data from experiments using all-*trans* RA followed by a Bonferroni-Dunns *post-hoc* test as this data set failed normality. In addition, a t-test was performed on data from experiments using TTNPB and PA024 as these separate experiments consisted of only two data sets. In addition, all data sets performed with *Xenopus* were analyzed using a 1-way ANOVA followed by a Tukey-Kramer *post hoc*. In addition, the length of any given neurite emerging from the cell body was calculated by taking the mean length of all its branched processes. The mean neurite length for each cell was then calculated by averaging the lengths of all neurites for that cell. Results are expressed as mean ± standard error of the mean (SEM) and were deemed significant when $p<0.05$.

Chapter 3.1: Results

The role of the RXR and novel, non-chordate RAR in retinoic acid-mediated chemoattraction in the pond snail, *Lymnaea stagnalis*

In the CNS of adult *Lymnaea*, both all-*trans* RA and 9-*cis* RA isomers are found to be present, at relatively equal concentrations (Dmetrichuk *et al*. 2008). Furthemore, studies on the effects of RA on growth cone behaviour in our lab have previously shown that these two RA isomers exert very similar effects on the growth cones (Dmetrichuk *et al*. 2006; Dmetirchuk *et al*. 2008; Farrar *et al*. 2009). Both all-*trans* and 9-*cis* exert positive growth cone turning with very similar turning angles. Carter *et al*. (2010) previously investigated the potential role of the RXR in mediating the response of RA. They showed that the RXR pan-agonist, PA024, could mimic the effects of both all-*trans* and 9-*cis* RA and that the RXR agonist repsonse was blocked by the RXR antagonists, PA452 and HX531. This previous study did not however go as far as to test whether the RXR antagonist was able to block the growth cone turning responses to both all-*trans* and 9-*cis* isomers. Therefore, the first aim of my thesis was to determine the role that the RXR may play in growth cone behaviour induced by the known endognenous isomers, all-*trans* and 9-*cis* RA.

3.11 The RXR antagonist HX531 blocked 9-*cis* retinoic acid-induced positive growth cone turning.

As 9-*cis* RA is the natural ligand for the RXR in vertebrates and is known to also bind the RXR in invertebrates (Kastner *et al*. 1995; Nowickyj *et al*. 2008), I first sought to determine if the RXR plays a role in the growth cone turning induced by 9-*cis* retinoic acid. To test this, a local gradient of 9-*cis* retinoic acid (10^{-5}M) was applied to an advancing growth cone in the presence of either the RXR antagonist HX531 (10^{-6}M in bath), or in the presence of DMSO (0.01% in bath) as a vehicle control. Growth cone turns toward RA are depicted as a positive turning angle in the histograms whereas growth cone turns away from RA are depicted as a negative turning angle. Growth cones were found to turn toward the source of 9-*cis* retinoic acid in the presence of DMSOwith a mean turning angle of 33.9 $\pm$ 2.6° (n=8) (Figure 6Ai-iii). In contrast, when a gradient of 9-*cis* retinoic acid was applied in the presence of the RXR antagonist, HX531, positive growth cone turning was blocked, with a mean turning angle of -

20.1 ± 12.4° (n=7) (Figure 6Bi-iii). A histogram showing maximum turning angles of each individual growth cone in response to 9-*cis* retinoic acid in the presence of DMSO or HX531 is shown in Figure 6Aiii and Biii respectively. There was a significant difference between the mean turning angle towards 9-*cis* retinoic acid in DMSO compared to HX531 ($p<0.001$) (Figure 7). Because the RXR pan-antagonist HX531 was able to block 9-*cis* RA-induced growth cone turning, these data suggest that the RXR plays an important role in mediating 9-*cis* RA-induced growth cone guidance.

3.12 The RXR antagonist HX531 blocked all-*trans* retinoic acid-induced positive growth cone turning.

Although it is thought that 9-*cis* retinoic acid is the natural ligand for the RXR, at least in vertebrates, recent literature suggests all-*trans* RA may also bind to the RXR in invertebrates. Nowickyj *et al*. (2008) showed that both isomers, 9-*cis* and all-*trans* retinoic acid bind to the locust RXR with equal affinity. It is then possible that the RXR mediates the response to both naturally occuring isomers. Therefore I sought to determine whether this receptor also mediates all-*trans* RA-induced growth cone turning. I tested this idea by applying a local gradient of all-*trans* RA (10^{-5}M) to an advancing growth cone in the presence of either the antagonist vehicle control DMSO or the RXR antagonist HX531. Growth cones were found to turn toward the source of all-*trans* retinoic acid in the presence of DMSO (0.01% in bath) with a mean turning angle of 31.5 ± 2.4° (n=8) (Figure 8Ai-iii). In contrast, when a gradient of all-*trans* retinoic acid was applied in the presence of the RXR antagonist HX531 (10^{-6}M in bath), positive growth cone turning was blocked, with a mean turning angle of -5.8 ± 9.2° (n=11) (Figure 8Bi-iii). A histogram showing maximum turning angles of each individual growth cone in response to all-*trans* retinoic acid in the presence of DMSO or HX531 is shown in Figure 8Aiii and Biii. Although, four of eleven growth cones showed a positive turn toward RA in the presence of HX531, the overall mean turning angle was significantly less than the mean turning angle toward

all-*trans* retinoic acid in DMSO ($p<0.05$) (Figure 9). These data suggest that the RXR may also mediate the growth cone turning toward all-*trans* RA, as well as to 9-*cis* RA.

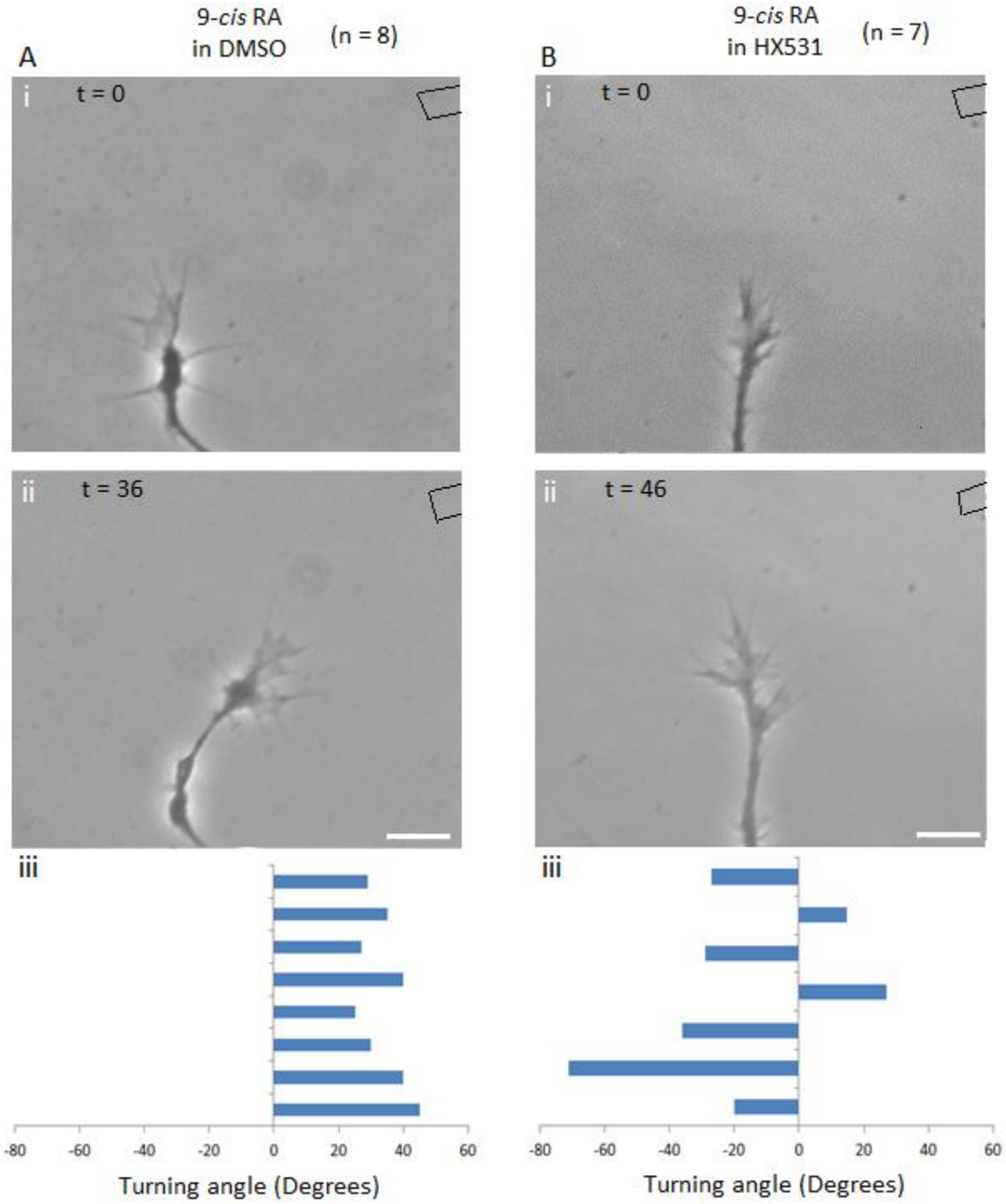

Figure 6. *Lymnaea* growth cones were attracted to a gradient of 9-*cis* retinoic acid and this response was blocked by the RXR antagonist, **HX531**. Representative images depicting the response of a growth cone to a local gradient of 9-*cis* retinoic acid in the absence (**Ai-ii**; DMSO) or presence of HX531 (**Bi-ii**). The pipette tip is outlined in the upper right hand corner of Ai-Bii. **Aiii and Biii**. Histograms showing the individual maximum turning angles toward the source of 9-*cis* retinoic acid in DMSO or HX531. (Times (t) are given in minutes. Scale bars = 15µm).

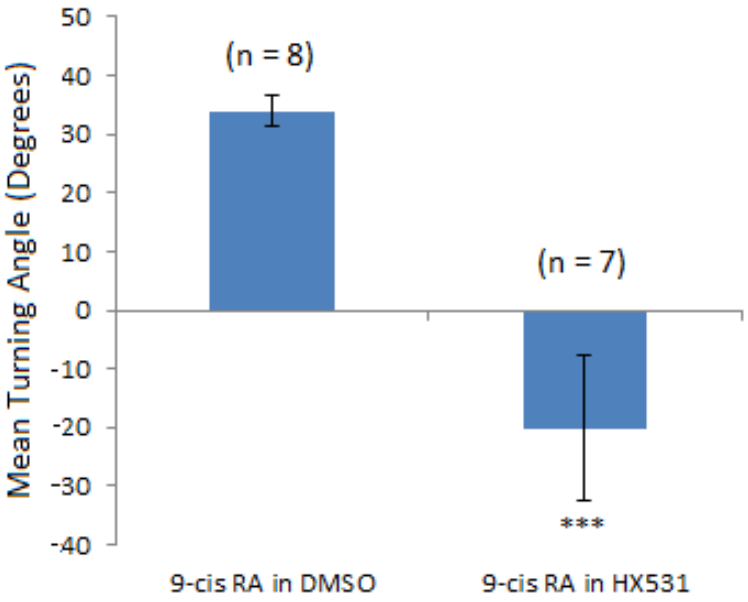

Figure 7. The RXR antagonist, HX531, blocked 9-*cis* retinoic acid induced positive growth cone turning. Summary graph showing the mean maximum turning angle exhibited by the growth cones in response to 9-*cis* retinoic acid in the presence of the vehicle control DMSO compared to the RXR antagonist HX531. A statistically significant difference is present between the two conditions, *** $p<0.001$, 1-way ANOVA. Error bars represent the standard error of the mean (SEM).

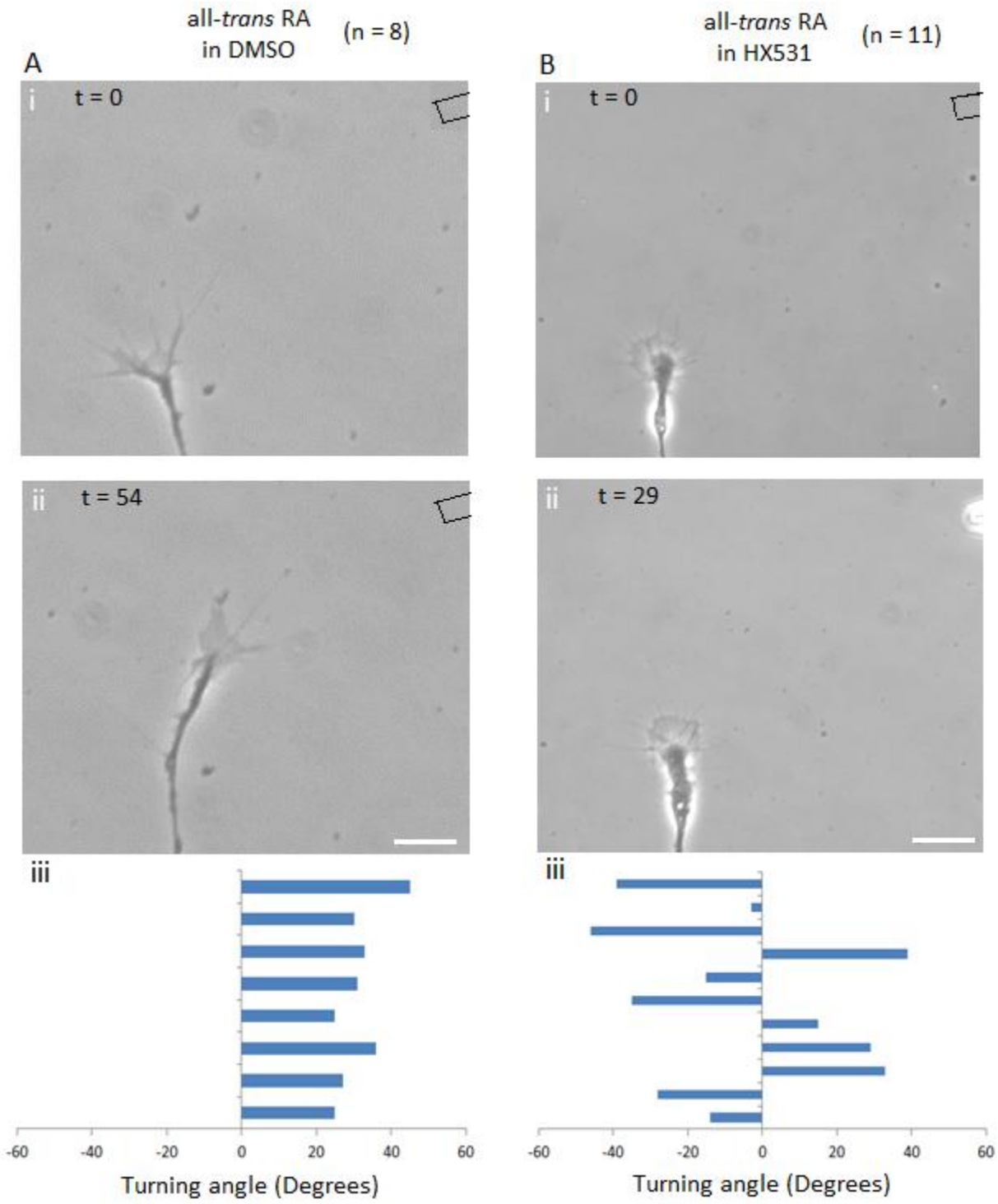

Figure 8. All-*trans* retinoic acid-mediated growth cone turning is blocked by the RXR antagonist, HX531. **Ai-ii.** Representative images depicting the turning response of a growth cone in response to a local gradient of all-*trans* retinoic acid in the presence of the vehicle control DMSO. **Bi-ii.** The RXR antagonist HX531 blocked all-*trans* retinoic acid-induced growth cone turning. The pipette tip is shown in upper right hand corner in Bii and the approximate location is illustrated otherwise. **Aiii and Biii**. Histograms showing the maximum turning angles of each individial growth cone toward all-*trans* RA in either DMSO or HX531. (Times (t) are given in minutes. Scale bar = 15µm).

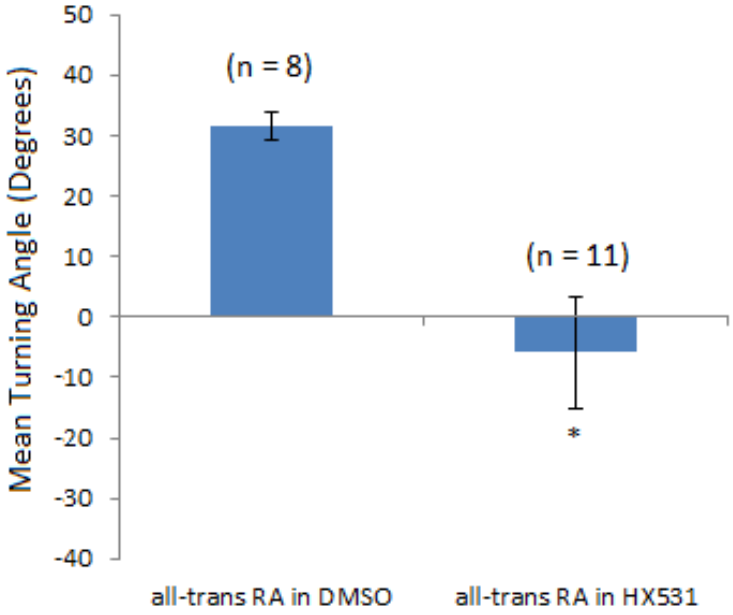

Figure 9. The RXR antagonist blocked all-*trans* retinoic acid induced positive growth cone turning. Summary graph showing the mean turning angles exhibited by the growth cones in response to all-*trans* retinoic acid in the presence of the vehicle control, DMSO (0.01%), or the RXR antagonist, HX531 (10^{-6}M). A statistically significant difference is present between the two conditions, * $p<0.05$, ANOVA on ranks. Error bars represent the standard error of the mean (SEM).

3.13 RXR is present in the growth cones of cells regardless of their response to a gradient of either retinoic acid or the RXR agonist, PA024.

Carter *et al*. (2010) previously showed the presence of RXR in growth cones of PeA neurons and evidence from neurites isolated from the cell body, suggests that local activation of the RXR (by RA or by an RXR agonist) is sufficient to produce the growth cone response. However, this article also showed that a small number of growth cones (7 of 102) did not exhibit RXR immunoreactivity. These data raise the possibility that in this study, some growth cones may not have responded to the retinoids due to the absence of the RXR. To rule out this possibility, growth cones that failed to respond to RA in the presence of HX531 (RXR antagonist) were immunostained to confirm the presence of RXR. Growth cones that turned toward the RXR pan-agonist, PA024, were used as positive controls. Growth cones that showed positive turning to PA024 did indeed show immunoreactivity for RXR (n = 8; Figure 10Ai-ii) as indeed did those growth cones that failed to respond to all-*trans* RA (n = 4) or 9-*cis* RA (n = 4) in the presence of HX531 (Figure 10Bi-ii). In contrast, cells used as controls that were labelled with only the secondary antibody showed no immunofluorescence (n = 10; data not shown). These data suggest that the lack of a growth cone turning response is not because the RXR is absent from the growth cone, but due to the presence of the RXR pan-antagonist, HX531, which is blocking the positive growth cone turning response exerted by RA (n = 16).

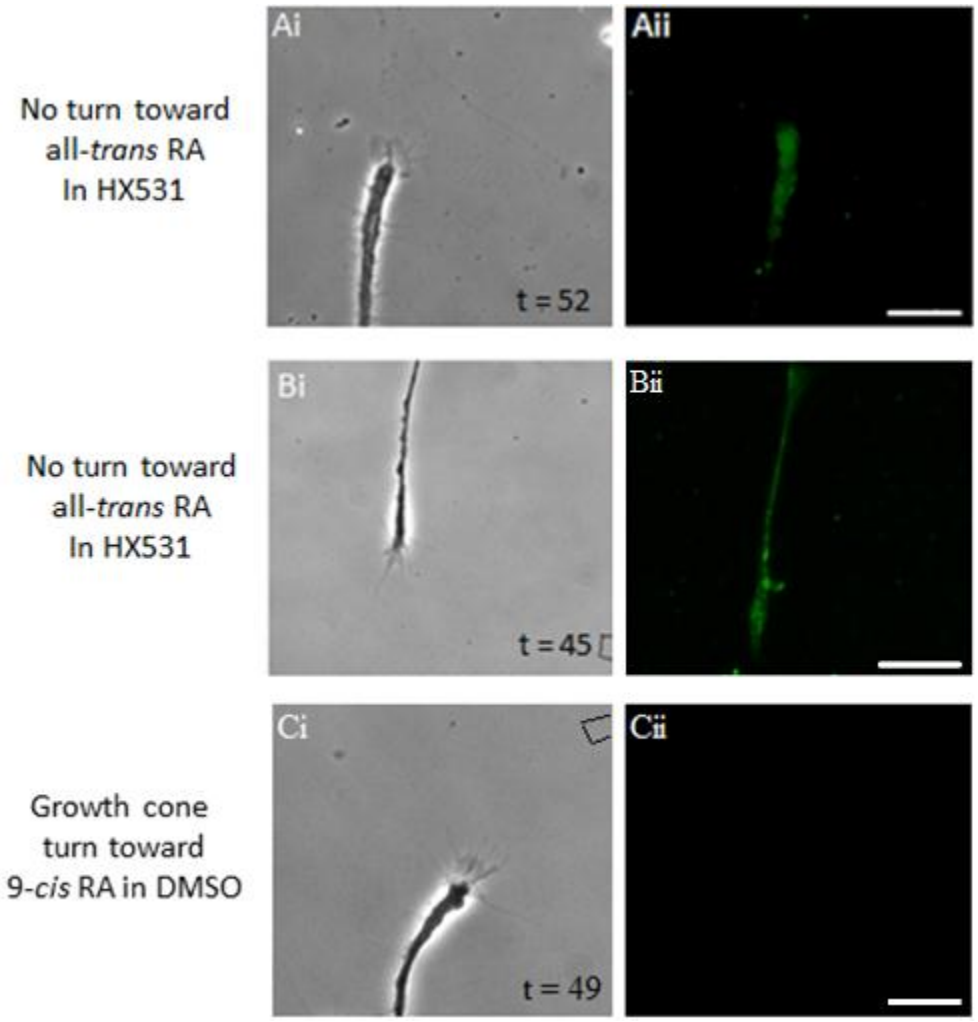

Figure 10. ***Lymnaea*** **RXR is present in growth cones that do not respond to all-*trans* RA in the presence of HX531.** Phase contrast images showing the endpoint of the growth cone assays prior to cell fixing (**Ai-Bi**). Immunostaining shows the RXR (green) to be present in the neurite and growth cone of a cell which turned towards a gradient of the RXR agonist PA024 in the vehicle control DMSO (**Aii**; n = 8)) and a growth cone which did not turn towards a gradient of all-*trans* retinoic acid in the presence of the RXR antagonist HX531 (**Bii**; n = 4). Note the lack of immunostaining in the control which lacked the primary antibody **(Ci-ii).** (Times (t) are given in minutes. Scale bars = 15 µm).

The data shown thus far suggest that both all-*trans* and 9-*cis* RA isomers might act via the RXR to mediate growth cone turning. However, it is known from vertebrate binding studies that both these RA isomers also bind to the RAR. Until recently, no RARs had been identified in non-chordates. However, Carter (2011) recently cloned the first non-chordate RAR, showing its expression in embryo, adult CNS and regenerating neurons in culture. Interestingly, like the RXR, RAR also showed non-nuclear distribution in adult neurons, with RAR immunoreactivity found in neurites and growth cones. This evidence raises the possibility that all-*trans* and 9-*cis* RA might also exert their chemoattractive effects to produce growth cone turning via the RAR. It was therefore my next aim to determine the role of this novel RAR in chemoattraction and to determine if it mediates retinoic acid-induced growth cone turning in *Lymnaea* neurons.

3.14 The RAR antagonist LE540 blocked all-*trans* retinoic acid-induced growth cone turning.

No previous studies have examined the role of RAR in growth cone turning, therefore I assessed the role of the novel, non-chordate RAR in RA-mediated chemoattraction. First, I set out to determine whether this receptor mediates RA-induced growth cone turning by performing a growth cone assay and applying a local gradient of all-*trans* retinoic acid (10^{-5}M in pipette) in the presence of the RAR pan-antagonist, LE540 (10^{-6}M in bath). Previous evidence from Carter (2011) shows that this concentration of LE540 exerts effects on *Lymnaea* development. When all-*trans* retinoic acid was applied in the presence of the RAR antagonist LE540, positive growth cone turning was blocked, producing a mean turning angle of -11 $\pm$ 5.8° (n=10) (Figure 11Bi-iii). The mean turning angle for all-*trans* retinoic acid in the presence of DMSO (0.01% in bath) was 31.5 $\pm$ 2.4° (n=8) (Figure 11Ai-iii). Histograms illustrating the maximum turning angle of each individual growth cone in response to all-*trans* retinoic acid in the presence of DMSO or LE540 are shown in Figure 11Aiii and Biii. Note that the control data set for all-*trans* RA applied in the presence of DMSO is the same as that shown in Figures 8 and 9 and is shown again here as a comparison to the antagonist data set. As stated previously, in the presence of DMSO, 8 of 8

growth cones turned toward all-*trans* RA with a mean turning angle of 31.5 ± 2.4° (Figure 9). However, in the presence of LE540, only 2 of 10 showed positive turning and the overall mean turning angle of -11 ± 5.7° was significantly less compared to all-*trans* RA in DMSO ($p<0.05$) (Figure 12).

3.15 9-*cis* retinoic acid-induced growth cone turning was blocked by the RAR antagonist LE540.

Previous evidence has shown that 9-*cis* RA binds to both the RXR and RAR in vertebrates with almost equal affinity (Maden, 2007). Therefore, I next aimed to determine if the RAR mediates 9-*cis* retinoic acid-induced turning. Interestingly, when 9-*cis* retinoic acid was applied in the presence of the RAR antagonist LE540, positive growth cone turning was once again blocked (mean turning angle of -28.4 ± 5.9° (n=11)) (Figure 13Bi-iii). These data were compared to 9-*cis* retinoic acid in the presence of DMSO which elicited a mean turning angle of 33.9 ± 2.6° (n=8) (Figure 13Ai-iii). A histogram illustrating the maximum turning angles of each individual growth cone in response to 9-*cis* retinoic acid in the presence of DMSO or LE540 is shown in Figure 13Aiii and Biii. Note that although a different representative image has been shown in Figure 13, the control growth cone turning data in response to 9-*cis* RA in DMSO are the same as those presented in Figures 6 and 7 and are shown again here for the purpose of comparison. There was a significant difference between the mean turning angle towards 9-*cis* retinoic acid in DMSO compared to LE540 ($p<0.001$) (Figure 14). In summary, the RAR pan-antagonist, LE540, blocked positive growth cone turning elicited by both all-*trans* and 9-*cis* retinoic acid, suggesting a role for the RAR, in addition to the RXR, in RA-mediated chemoattraction.

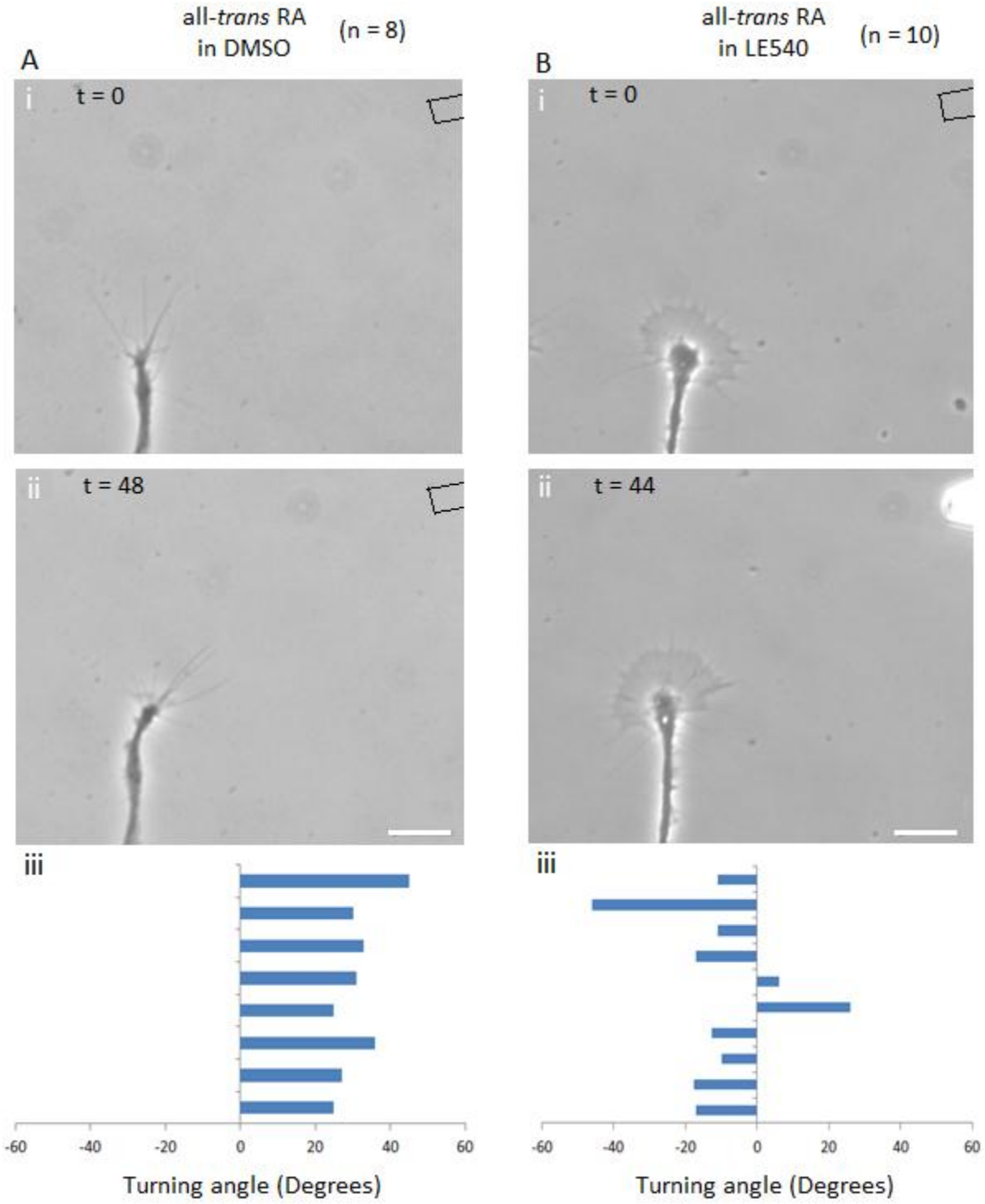

Figure 11. *Lymnaea* growth cones were attracted to a gradient of all-*trans* retinoic acid and this response was blocked by the RAR pan-antagonist, LE540. (**Ai-ii**) Representative images depicting the turning response of a growth cone to a local gradient of 9-*cis* retinoic acid in the vehicle control DMSO. This response was inhibited in the presence of the RAR antagonist LE540 (**Bi-ii**). The pipette tip is outlined in the upper right hand corner of Ai-ii and Bi. **Aiii and Biii**. Histograms showing the individual maximum turning angles toward the source of retinoic acid. (Times (t) are given in minutes. Scale bars = 15µm). Note that the same control data for DMSO were used for comparison in this case as in Figures 8 and 9.

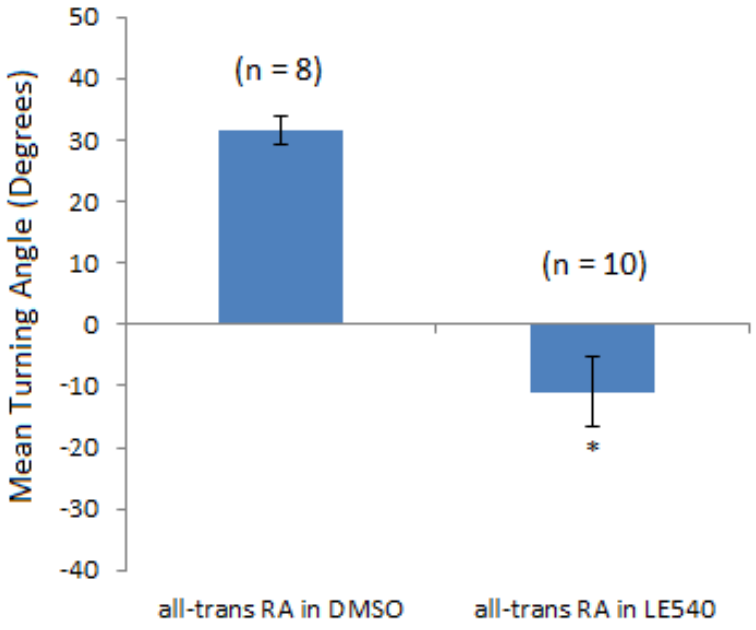

Figure 12. The RAR antagonist, LE540 blocked all-*trans* retinoic acid-induced positive growth cone turning. Summary graph showing the mean maximum turning angle exhibited by the growth cones in response to all-*trans* retinoic acid in the presence of the RAR antagonist LE540 compared to vehicle control (DMSO). A statistically significant difference was present between the two conditions, * $p<0.05$, ANOVA on ranks. Error bars represent the standard error of the mean (SEM).

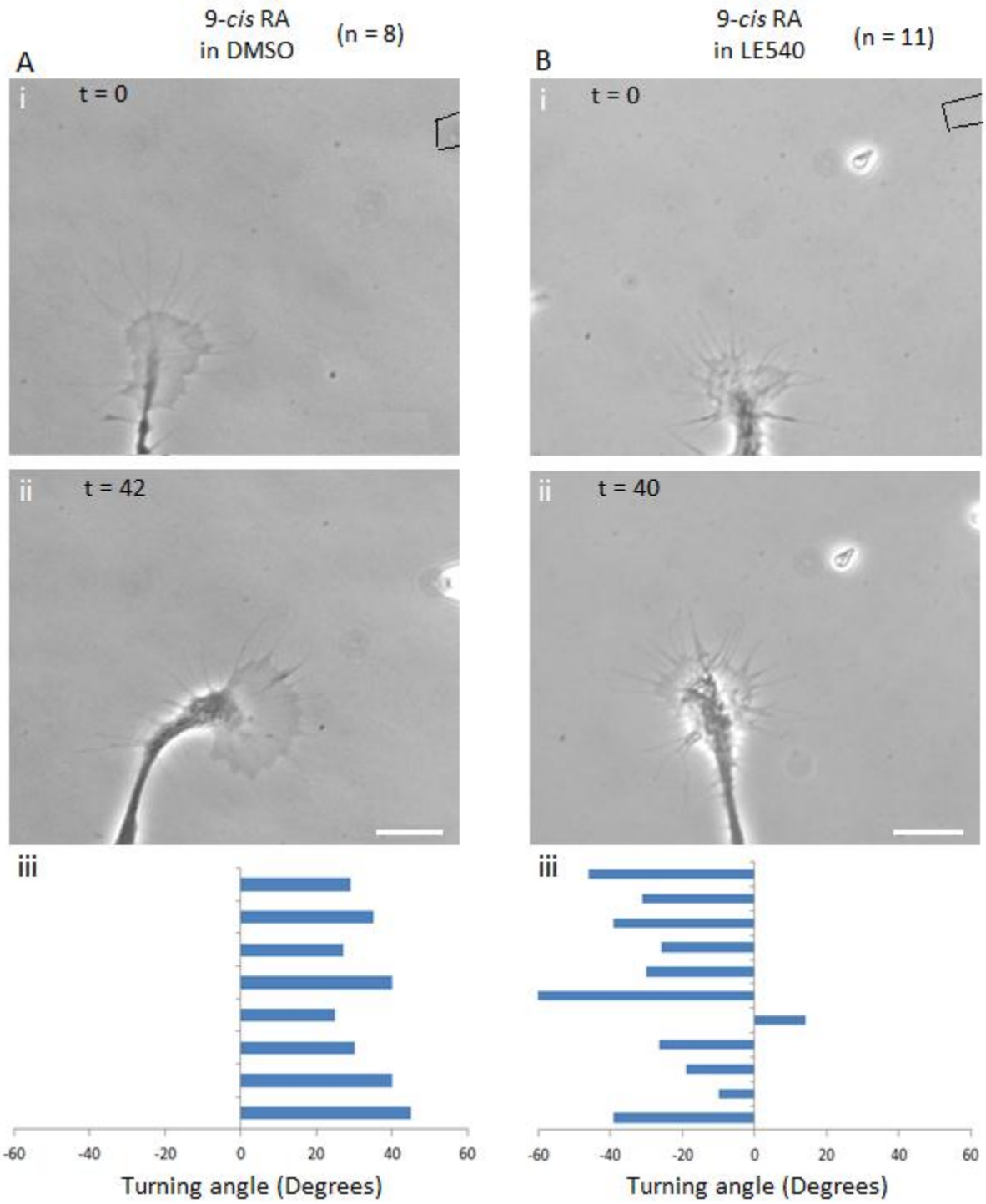

Figure 13. Positive growth cone turning responses to a gradient of 9-*cis* retinoic acid were blocked by the RAR antagonist, LE540. (**Ai-ii**) Representative images depicting the turning response of a growth cone to a local gradient of 9-*cis* retinoic acid in the vehicle control DMSO. This response was blocked in the presence of the RAR antagonist LE540 (**Bi-ii**). Pipette depicted in the upper right hand corner of **Ai** and **Bi**. **Aiii and Biii**. Histograms showing the individual maximum turning angles toward the source of retinoic acid. (Times (t) are given in minutes. Scale bars = 15µm).

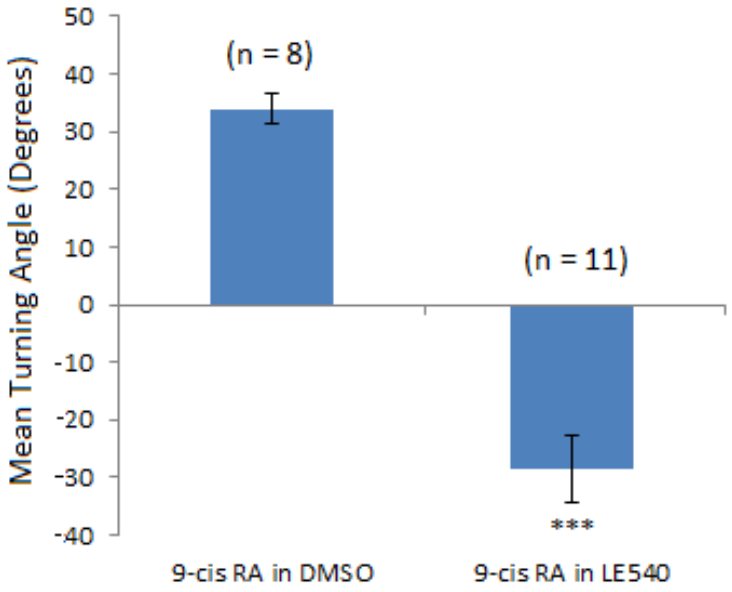

Figure 14. The RAR antagonist, LE540 blocked 9-*cis* retinoic acid-induced positive growth cone turning. Summary graph showing 9-*cis* retinoic acid-induced mean turning angles of growth cones in the presence of the vehicle control DMSO or the RAR antagonist LE540. The overall mean turning angle elicited by 9-*cis* RA in the presence of LE540 was significantly less compared to 9-*cis* RA in DMSO. *** $p<0.001$, 1-way ANOVA. Error bars represent the standard error of the mean (SEM).

3.16 The RAR antagonist does not block the RXR agonist, PA024, induced growth cone turning.

The data shown above suggest the *Lym*RAR may play a role in the RA-induced growth cone turning response. However, *Lym*RAR shows less similarity to vertebrate RARs (~58% homology in LBD) than the *Lym*RXR does to vertebrate RXRs (~80% homology in LBD). This raises the possiblity that the vertebrate RAR pan-antagonist, LE540, is not selective for *Lym*RAR and as this is the first non-chordate RAR cloned, no binding assays have yet been performed. My next aim was therefore to test the selectivity of the pan-RAR anatagonist, LE540, by testing its effects on the growth cone turning response to the applied RXR pan-agonist, PA024. Carter *et al*. (2010) previously showed that RXR pan-agonist turning response was blocked by the RXR anatgonist, but the RAR antagonist has not been tested previously. Furthermore, evidence indicates that LE540 can also bind RXRs, but is less able to inhibit these compared to RARs (Umemiya, 1997). The RAR pan-antagonist, LE540, was designed against the vertebrate RAR and therefore the selectivty for the *Lymnaea* RAR is unknown. Therefore, in order to investigate potential non-selective actions of LE540 or cross-reactivty with RXR, I examined whether the RAR-selective antagonist, LE540, is able to block a selective RXR agonist, PA024-induced growth cone turning.

A local gradient of PA024 (10^{-5}M in pipette) was applied to an advancing growth cone and it induced positive growth cone turns with a mean turning angle of 25.3 $\pm$ 4.4° (n=9) (Figure 15Ai-iii). Likewise, when PA024 was applied in the presence of the RAR antagonist LE540, the RXR agonist was still able to induce a positive turning angle with a mean angle of 24.7 $\pm$ 5.5° (n=11) (Figure 15Bi-iii). Histograms illustrating the maximum turning angle of each individual growth cone in response to PA024 in the presence of either DMSO or LE540, are shown in Figure 15Aiii and Biii. There was no significant difference between the application of PA024 alone or in the presence of LE540 (Figure 16). These data suggest that the RXR agonist, PA024, was able to induce growth cone turning in the presence of the RAR antagonist, LE540, which

suggests the binding of these chemicals to their respective receptors may be relatively selective (though binding studies would be required to verify this statement).

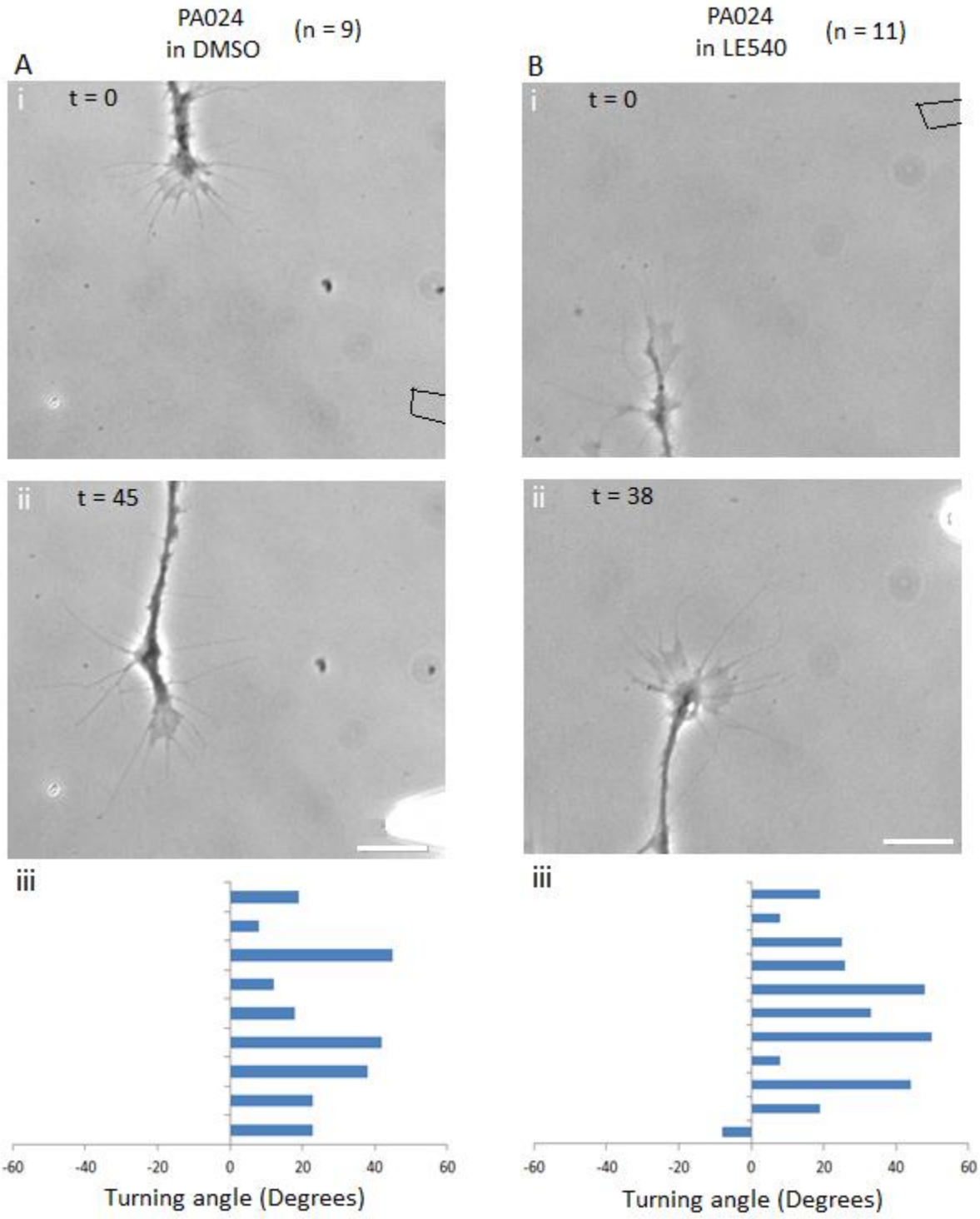

Figure 15. Intact growth cones were attracted to a gradient of the RXR agonist PA024 in the presence of the RAR antagonist LE540. (**Ai-ii**) Representative images depicting the turning response of a growth cone to a local gradient of PA024 in the presence of DMSO or the RAR antagonist LE540 (**Bi-ii**). The pipette tip is shown in the bottom (Ai-Aii) and upper (Bi-Bii) right hand corner. **Aiii and Biii**. Histograms showing the individual maximum turning angles toward PA024. (Times (t) are given in minutes. Scale bars = 15µm).

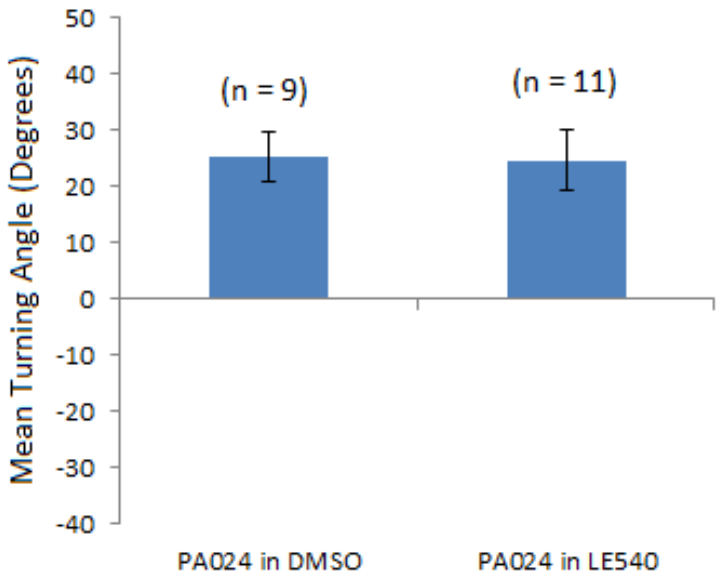

Figure 16. The RAR antagonist LE540 did not block the RXR agonist, PA024-induced growth cone turning. Summary graph showing nearly identical mean turning angles exhibited by growth cones in response to PA024 in the presence of DMSO or the RAR antagonist LE540. No statistically significant difference was found between the two conditions ($p>0.05$, t-test). Error bars represent the standard error of the mean (SEM).

3.17 The RAR agonist TTNPB does not induce positive growth cone turning

The inability of the RAR antagonist LE540 to block the RXR agonist, PA024-induced growth cone turning, suggests that the two receptors may be acting independently from one another to mediate this response, but more specifically provides evidence that less cross-reactivity of agonists and antagonists is present. Therefore, I next aimed to determine whether the RAR-selective agonist, TTNPB (10^{-5}M in pipette) was sufficient to elicit a positive growth cone turning response when compared to the focal application of the vehicle control DMSO (0.1% in pipette). However, it should be noted that TTNPB was designed against the vertebrate RAR and has previously been shown to have similar IC_{50}s to all-*trans* RA, the natural ligand for RARs, based on competitive binding assays in mice (Pignatello *et al*. 1999). In addition, Pignatello *et al*. (1999) showed that TTNPB had no appreciable affinity for any of the RXR subtypes. A local gradient of DMSO was applied to an advancing growth cone and it was determined that this control assay did not induce positive growth cone turning (mean turning angle of -8.3 $\pm$ 6.4°; n=10) (Figure 17Ai-iii). When the RAR agonist TTNPB was applied in the same fashion, it also did not induce positive turning (mean turning angle of -7.1 $\pm$ 7.7°; n=15) (Figure 17Bi-iii). A histogram illustrating the maximum turning angle of each individual growth cone in response to DMSO or TTNPB is shown in Figure 17Aiii and Biii. There was no significant difference in mean turning angles between the application of DMSO and TTNPB (Figure 18). This evidence suggests that either activation of the RAR is not sufficient to induce growth cone turning or that TTNPB is not activating the *Lymnaea* RAR. Looking at the individual growth cone turning angles however, TTNPB appeared to elicit a larger variance in turning angles whereas DMSO controls showed little or no response either towards or away from the DMSO source (with the exception of 1 growth cone). We do not currently know enough about the *Lymnaea* RAR sequence to predict that TTNPB will bind to it, therefore binding studies will be required to fully elucidate the effects of TTNPB.

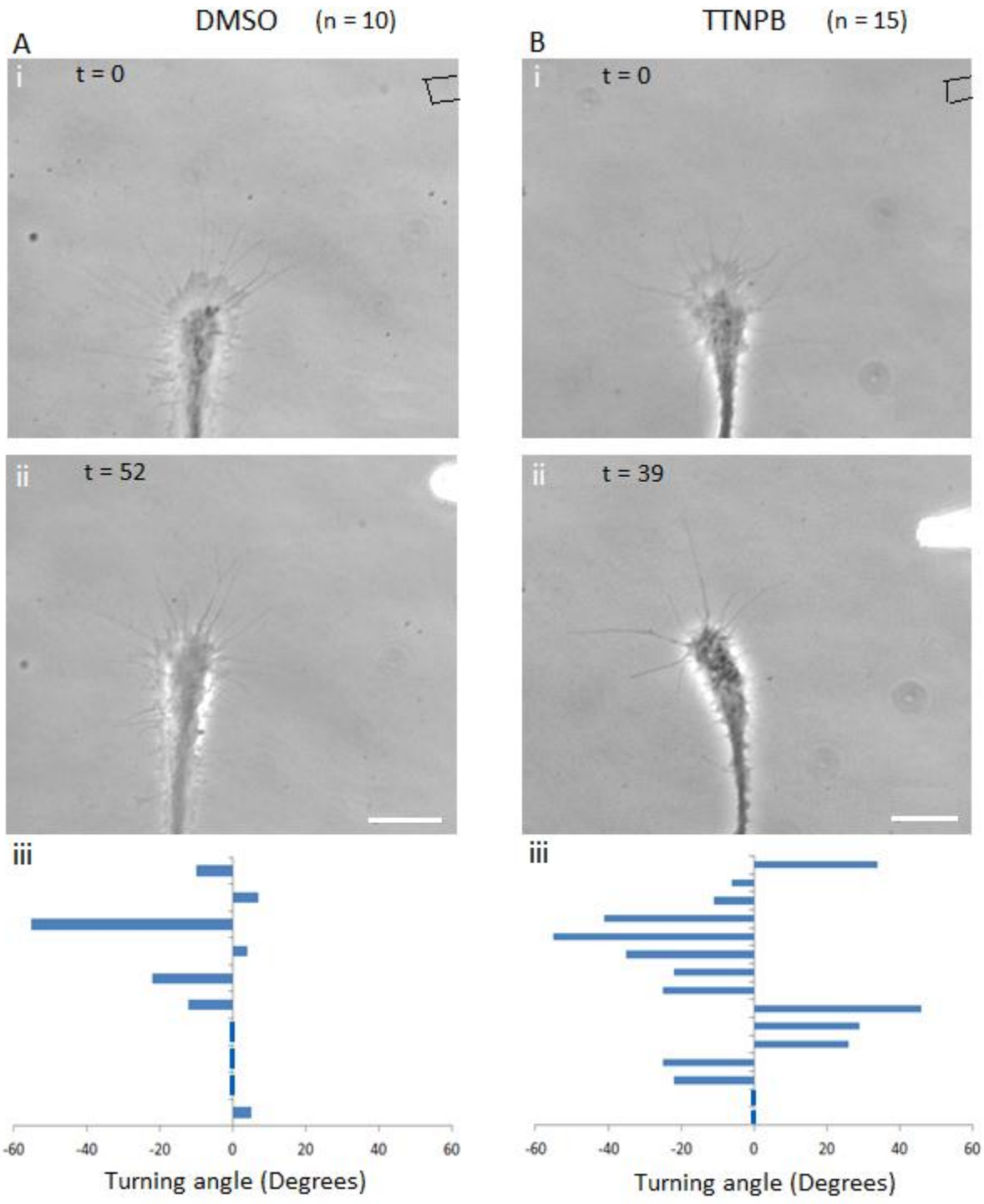

Figure 17. Pedal A growth cones did not turn toward the RAR agonist TTNPB. Representative images depicting the turning response of a growth cone in response to a local gradient of DMSO (**Ai-ii**) or the RAR agonist TTNPB (**Bi-ii**). **Aiii and Biii**. Histograms showing the individual maximum turning angles toward or away from DMSO or TTNPB. The pipette tip is illustrated in the upper right hand corner (Ai-Bii). (Times (t) are given in minutes. Scale bar = 15µm).

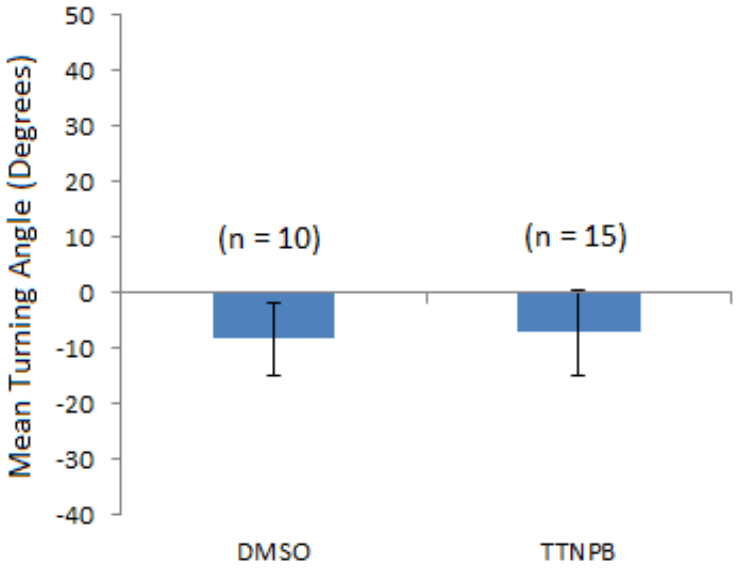

Figure 18. The RAR agonist TTNPB was not able to induce positive growth cone turning compared to the DMSO control. Summary graph showing the mean maximum turning angle exhibited by the growth cones in response to DMSO or the RAR agonist TTNPB. No statistical significance was found between the mean turning angles elicited by DMSO compared to TTNPB ($p>0.05$, t-test). Error bars represent the standard error of the mean (SEM).

4.18 Summary

The data shown here demonstrated that both the RXR and the newly cloned RAR are involved in growth cone turning in response to both endogenous RA isomers. I've also demonstrated that the lack of a turning response by some growth cones to a gradient of the RXR pan-agonist, PA024, was not due to the absence of the RXR in these particular growth cones. Furthermore, I've shown that the RXR pan-agonist and RAR pan-antagonist appear to have little cross-reactivity with the opposing retinoid receptor as PA024-induced growth cone turning was retained in the presence of LE540. Finally, I've shown that focal application of the RAR pan-agonist, TTNPB, is insufficient to elicit the positive growth cone turning that is seen with RA and PA024.

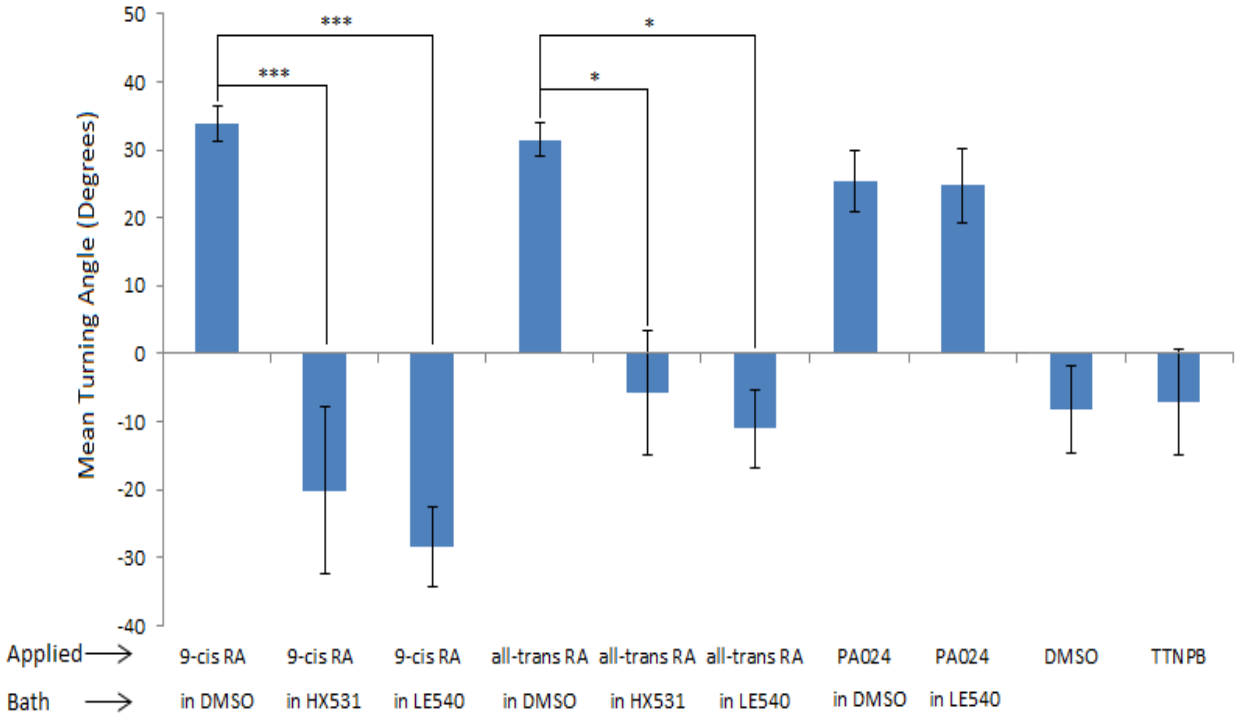

Figure 19. Summary graph showing the mean maximum turning angles of growth cones in various conditions. Statistical comparisons are made to the DMSO vehicle control groups. Error bars represent standard error of the mean (SEM). HX531 – RXR antagonist, LE540 – RAR antagonist, PA024 – RXR agonist, TTNPB – RAR agonist.* $p<0.05$, *** $p<0.001$.

Chapter 3.2: Results

The conservation of retinoic acid's neurotrophic and chemotropic role in the South African clawed frog, *Xenopus laevis*

3.21 Retinoic acid acts a neurotrophic factor for *Xenopus* embryonic spinal cord neurons, inducing significant neurite outgrowth of cultured neurons.

As discussed above, the role of retinoic acid has been well documented in *Lymnaea stagnalis* in terms of its neurotrophic and chemotropic activities *in vivo* and *in vitro* (Dmetrichuk *et al*. 2006, Farrar *et al*. 2009 and Carter *et al*. 2010). However, our lab has previously shown that all-*trans* retinoic acid is capable of inducing significant neurite outgrowth from cultured newt spinal cord explants (Dmetrichuk *et al*. 2005). In addition, retinoic acid has previously been implicated in axonal guidance in cultured chick neural tube cells (Maden *et al*. 1998). Based on this evidence, we wanted to determine if RA acts as a chemotropic molecule to induce individual growth cone turning in vertebrate neurons. We chose to perform these growth cone assays experiments with the South-African clawed frog, *Xenopus laevis*. These embryos are easily obtained and previous literature has shown the capacity for isolation of the neural tube and dissociation of spinal cord neurons to allow for growth cone assays to be performed on individual growth cones. Furthermore, RA has not previously been shown to exert effects in these neurons. However, I also wished to determine if RA could exert neurotrophic effects on embryonic spinal cord neurons first, as has been shown in many other vertebrate embryonic neurons. Hence, my first aim was to look at the potential neurotrophic effects of both isomers, as has been shown previously in the newt (Dmetrichuk *et al*. 2005; Rand, 2009, Zeglinski, 2008) on cultured embryonic spinal cord neurons from the stage 22 *Xenopus* embryo.

Spinal cord neurons were cultured from the stage 22 embryo and plated in DM. Retinoic acid (all-*trans* and 9-*cis*) was added to the media at 10^{-7}M (bath concentration) while EtOH, acting as the vehicle control, was added at 0.001% (bath concentration). Ethanol induced a mean neurite length of 20 μm $\pm$ 1.2 (n=192) (Figure 20A). In contrast, all-*trans* retinoic acid induced a mean neurite length of 34.5 μm $\pm$ 1.6 (n=376) (Figure 20B). In addition, 9-*cis* retinoic acid induced a mean neurite length of 33.2 μm $\pm$ 2.8 (n=113) (Figure 20C). Statistical analysis revealed that both isomers of retinoic acid induced significantly longer mean neurite lengths of

cells identified as neurons compared to the vehicle control ($p<0.05$; Figure 20D). In addition, the number of neurons which survived and extended neurites (and therefore were capable of being identified as neurons based on morphology), in all-*trans* retinoic acid, 9-*cis* retinoic acid or EtOH was 84, 77 and 39 respectively, over the same number of dishes.

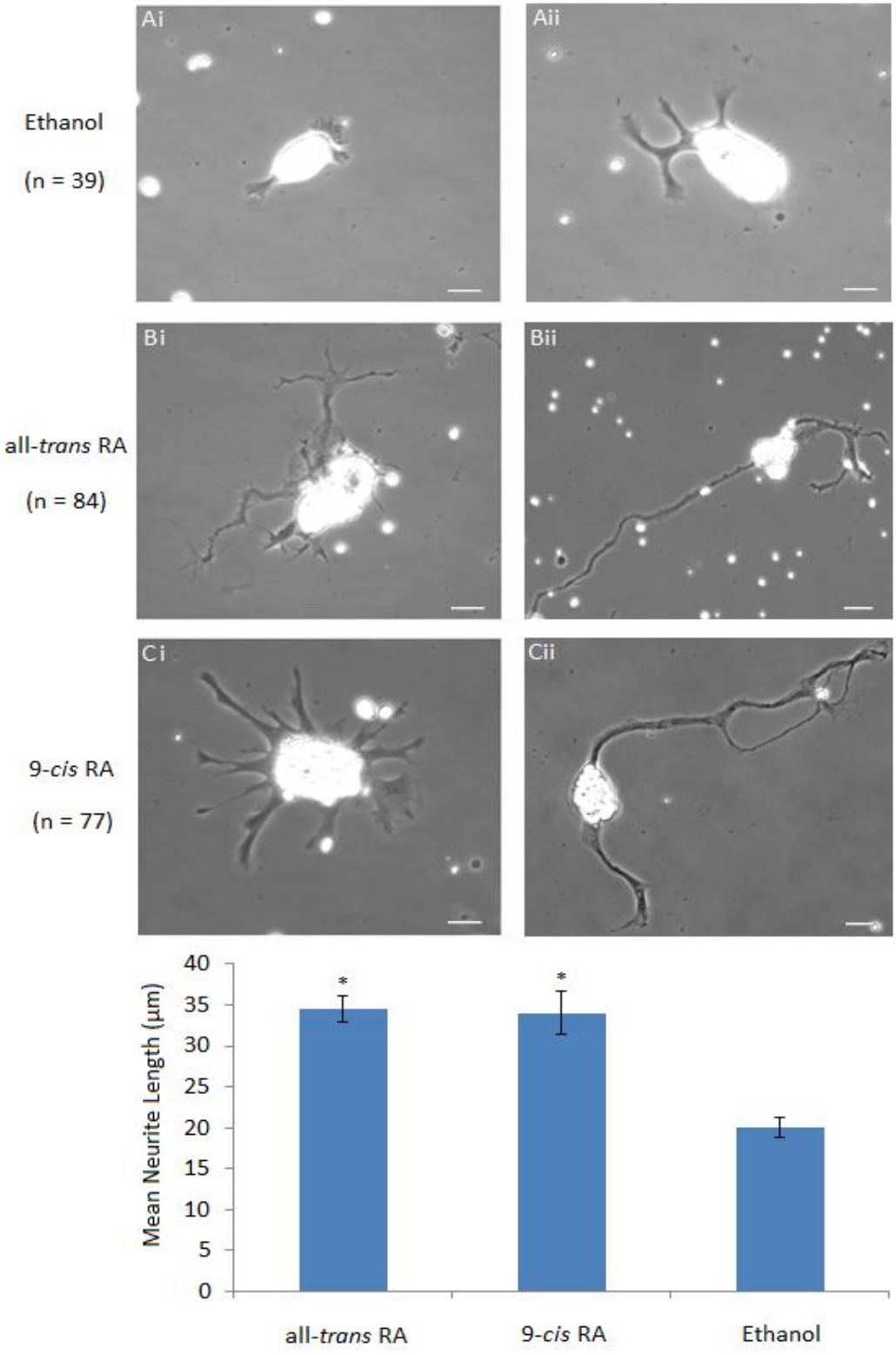

Figure 20. Cultured *Xenopus* embryonic spinal cord neurons had significantly more outgrowth in the presence of either all-*trans* or 9-*cis* retinoic acid when compared to the ethanol control. Representative images of *Xenopus* embryonic spinal cord neurons cultured in EtOH (0.001%; **Ai-ii**), all-*trans* retinoic acid (10^{-7}M; **Bi-ii**) or 9-*cis* retinoic acid (10^{-7}M; **Ci-ii**). **D)** Histogram showing mean neurite length of individual cultured embryonic spinal neurons in each condition indicating a trophic role for RA. Scale bar = 15 µm. Error bars represent standard error of the mean (SEM). *p<0.05, 1-way ANOVA.

3.22 RARβ mediates the chemoattractive activity of all-*trans* retinoic acid for *Xenopus* embryonic spinal cord cells *in vitro*.

The data shown in Figure 20 demonstrate RA's neurotrophic conservation in these vertebrate neurons. Therefore, I next aimed to determine whether RA could act as a chemotropic molecule and induce growth cone turning of cultured spinal cord neurons. After approximately 18-20 hours *in vitro* in 10^{-7}M all-*trans* RA (bath concentration), spinal cord cultures were checked for outgrowth and neurons were identified based on their morphology (previously described in the Methods, Figure 5). A local gradient of EtOH (0.1% in pipette) was applied using a pressure pipette to an advancing growth cone and did not induce growth cone turning (mean turning angle of 1.1 $\pm$ 4.6°) (n=7) (Figure 21Ai-iii). In contrast, focal application of all-*trans* retinoic acid (10^{-5}M) elicited a positive growth cone turn with a mean turning angle of 32.9 $\pm$ 3.3° (n=8) (Figure 21Bi-iii). In order to assess which receptor may mediate this response to all-*trans* RA, I first decided to use the RARβ-selective antagonist, LE135, as prevous data from our lab showed that this antagonist was able to block neurite outgrowth elicited by all-*trans* retinoic acid from cultured newt spinal cord explants (Dmetrichuk *et al*. 2005). Interestingly, when all-*trans* retinoic acid was applied in the presence of LE135, positive growth cone turning was blocked with a mean turning angle of -21.6 $\pm$ 4.6° (n=7) (Figure 21Ci-ii). Histograms showing maximum turning angles of individual growth cones in response to EtOH, all-*trans* retinoic acid alone or in the presence of LE135 are shown in Figure 21 Aiii-Ciii. When the overall mean turning angle elicited by EtOH was compared to all-*trans* RA, it was found that all-*trans* RA induced a significantly larger turning angle compared to EtOH ($p<0.001$) (Figure 23), and that this response to RA was signficantly reduced in the presence of LE135 ($p<0.001$) (Figure 23). Note that four growth cones assays were performed in DM alone (mean = 29.6°) and three growth cone assays were performed in the presence of DMSO (mean = 38.3°) suggesting DMSO had was not responsible for the decrease in mean turning angle in response to all-*trans* RA in the presence of LE135. These data show for the first time, that all-*trans* RA-induced growth cone

turning may be conserved in vertebrate neurons and suggest that RARβ may be mediating the all-*trans* RA-induced chemoattraction of *Xenopus* embryonic vertebrate neurons.

3.23 RXR mediates the growth cone turning induced by 9-*cis* retinoic acid of *Xenopus* embryonic spinal cord cells.

We next aimed to assess whether the RA isomer, 9-*cis* retinoic acid, was able to induce growth cone turning as it has previously been shown to do so in cultured *Lymnaea* neurons (Carter *et al*. 2010). As in the invertebrate model, when a gradient of 9-*cis* RA was applied, *Xenopus* growth cones turned towards the source of 9-*cis* RA with a mean turning angle of 34.8 $\pm$ 6.9° (n=8) (Figure 22Ai-ii). Because 9-*cis* RA is a natural ligand for the RXR (even though it also binds to RAR), we decided to assess the RXR's role in mediating this response to 9-*cis* RA. As hypothesized, when 9-*cis* RA was applied in the presence of the RXR antagonist, HX531, positive turning was blocked (mean turning angle of -8.3 $\pm$ 3.6°) (n=6) (Figure 22Bi-ii). Histograms showing maximum turning angles of individual growth cones in response to 9-*cis* retinoic acid alone or in the presence of HX531 is shown in Figure 22 Aiii-Biii. When taken together, the overall mean turning angle induced by 9-*cis* RA was significantly higher than focal application of EtOH ($p<0.001$). Furthermore, when the RXR antagonist, HX531, was present in the bath, 9-*cis* RA-induced growth cone turning was significantly reduced ($p<0.001$) (Figure 23). Note that four growth cones assays were performed in DM alone (mean = 27°) and four growth cone assays were performed in the presence of DMSO (0.01% in bath; mean = 42.5°) suggesting DMSO was not responsible for the decrease in mean turning angle in response to 9-*cis* RA in the presence of HX531. These data show for the first time, in a vertebrate model, the conservation of 9-*cis* retinoic acid-induced growth cone turning of *Xenopus* embryonic spinal cord neurons and that this response may be mediated by the RXR.

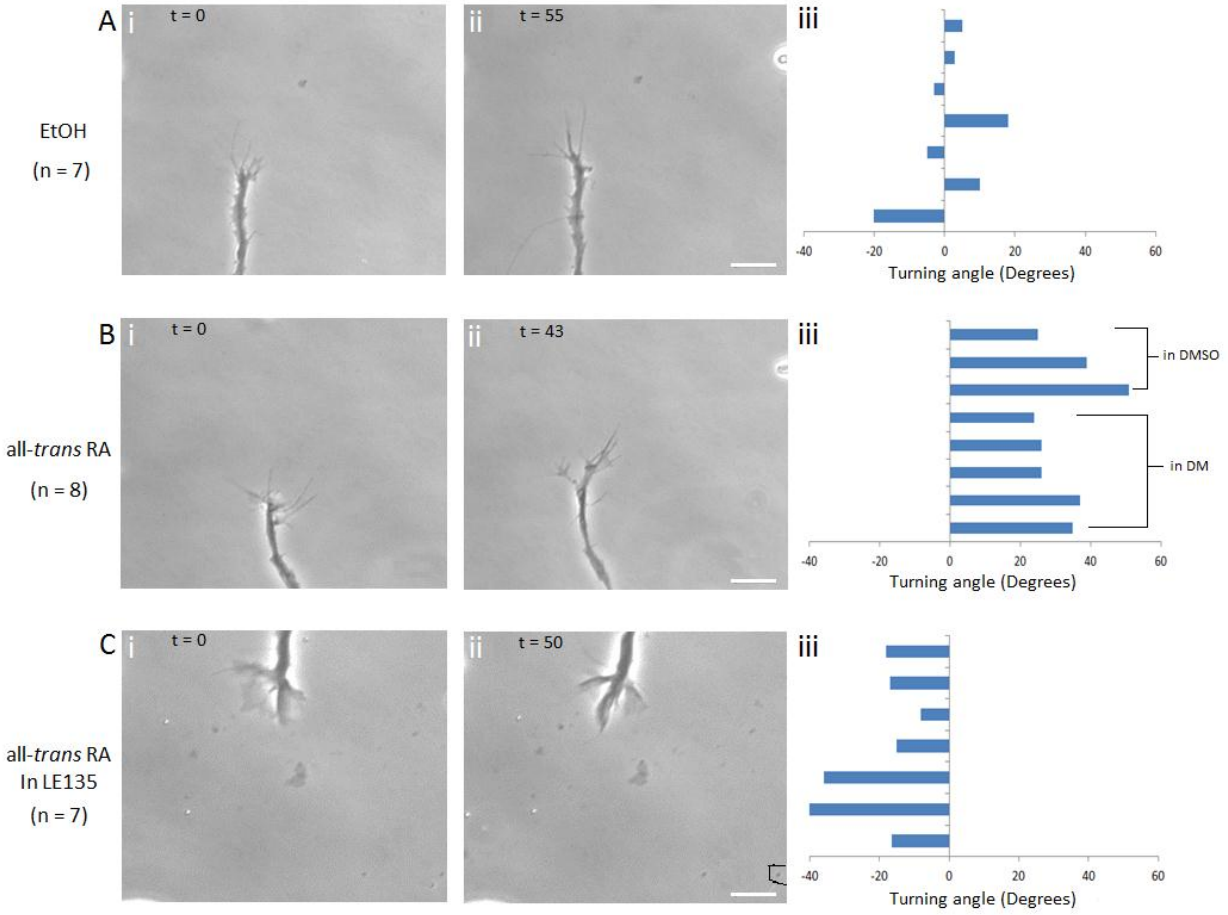

Figure 21. Cultured *Xenopus* embryonic spinal cord neuron growth cones were attracted to a gradient of all-*trans* retinoic acid and the RARβ antagonist LE135 blocked all-*trans* RA induced turning. Representative images depicting the turning response of a growth cone in response to a local gradient of EtOH (n=7) (**Ai-ii**), all-*trans* retinoic acid (n=8) (**Bi-ii**) and all-*trans* retinoic acid in the presence of the RARβ-selective antagonist LE135 (n=7) (**Ci-ii**). Histograms showing the individual maximum turning angles toward the pipette (**Aiii-Ciii**). The pipette tip location is illustrated on the right side of the representative images. Note that assays of three growth cones with all-*trans* RA applied were performed in the presence of DMSO (0.01%) and the other 4 were done in DM alone. (Times (t) are given in minutes. Scale bar = 15µm).

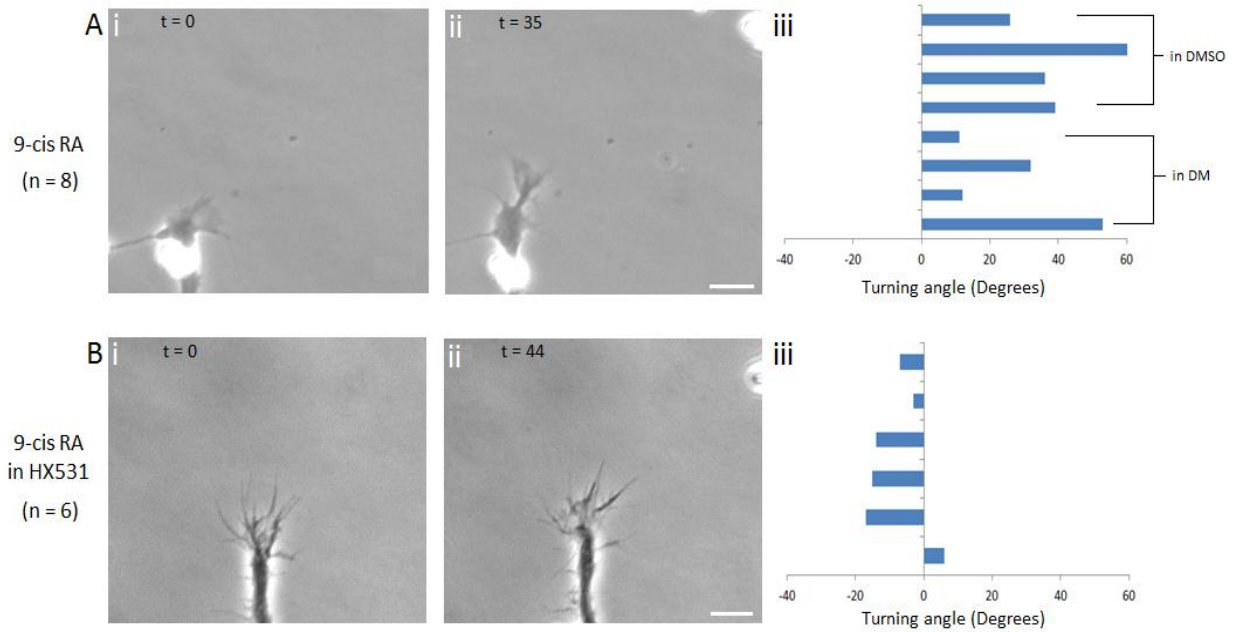

Figure 22. *Xenopus* growth cones were attracted to a gradient of 9-*cis* retinoic acid and the RXR antagonist HX531 blocked 9-*cis* RA induced turning. Representative images depicting the turning response of a growth cone to a local gradient of 9-*cis* retinoic acid (n=8) (**Ai-ii**) and 9-*cis* retinoic acid in the presence of the RXR antagonist, HX531 (n=6) (**Bi-ii**). Histograms showing the individual maximum turning angles toward the pipette (**Aiii-Biii**). The pipette tip location is illustrated in the upper right hand corner of the representative images. Note that four growth cones with 9-*cis* RA applied were performed in the presence of DMSO (0.01%). (Times (t) are given in minutes. Scale bar = 15µm).

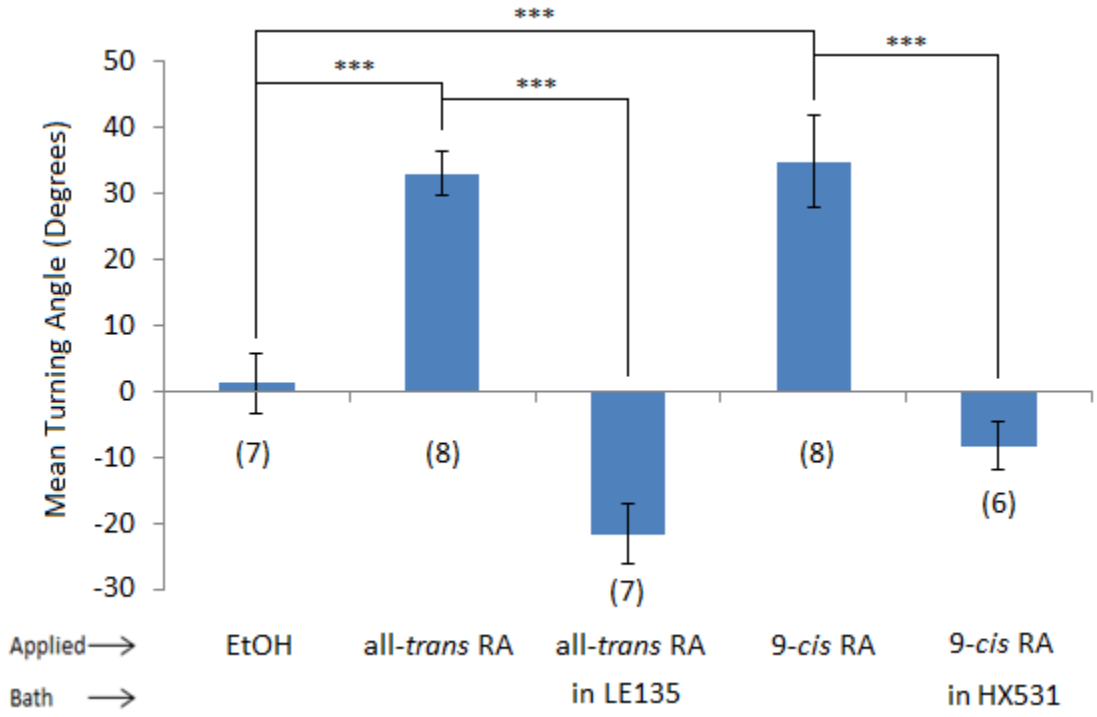

Figure 23. Summary diagram showing all *Xenopus* mean growth cone turning angle responses under various conditions. Statistical comparison are made between RA alone and the EtOH control group as well as between RA alone or in the presence of the retinoid receptor antagonists. LE135 – RARβ antagonist, HX531 – RXR antagonist.Error bars represent the standard error of the mean (SEM). *** $p<0.001$, 1-way ANOVA.

Farrar *et al*. (2009) and Carter *et al*. (2010) have previously shown a novel non-genomic role for retinoids in the invertebrate model, *Lymnaea stagnalis*, contrary to their classical role as nuclear receptors. They showed non-nuclear staining of the RXR in the cytoplasm, neurite and growth cone in these motorneurons. More recently, the novel non-chordate RAR has shown similar staining in cultured neurons (Carter, 2011). In addition, Carter (2011) also showed non-nuclear staining of a RARβ in the regenerating tail and spinal cord of the newt. However, non-nuclear staining of RARβ has never been shown in individual cultured vertebrate neurons. Furthermore, there are a few examples of RAR acting non-genomically in vertebrates (Canon *et al.* 2004; Liao *et al*. 2004; Poon and Chen, 2008). Therefore, my next aim was to investigate the localization of RARβ in embryonic spinal cord neurons from *Xenopus laevis* using immunocytochemistry.

3.24 Immunostaining revealed non-nuclear staining of the vertebrate RARβ in cultured embryonic spinal cord neurons.

Following growth cone assay experiments, the *Xenopus* spinal neurons were fixed and stained for RARβ and also for DAPI to visualize the cell nucleus. A control antibody solution, which lacked the primary antibody which would bind RARβ, was applied to some dishes (n = 6) to ensure there was no immunoreactivity from the secondary antibody alone. As expected no immunoreactivity was found in any of these dishes. For the first time, I showed non-nuclear staining of the RARβ in *Xenopus* embryonic spinal cord neurons, where the antigen was localized in the cytoplasm, neurite and growth cone of vertebrate neurons (n=10; Figure 24A and B), similar to that of *Lymnaea* neurons. This finding, combined with the blocking of all-*trans* retinoic acid-induced growth cone turning by LE135, suggest a novel non-genomic role for this receptor in mediating the effects of RA on outgrowth, survival and regeneration in the vertebrate CNS, although further experiments must be done to confirm this.

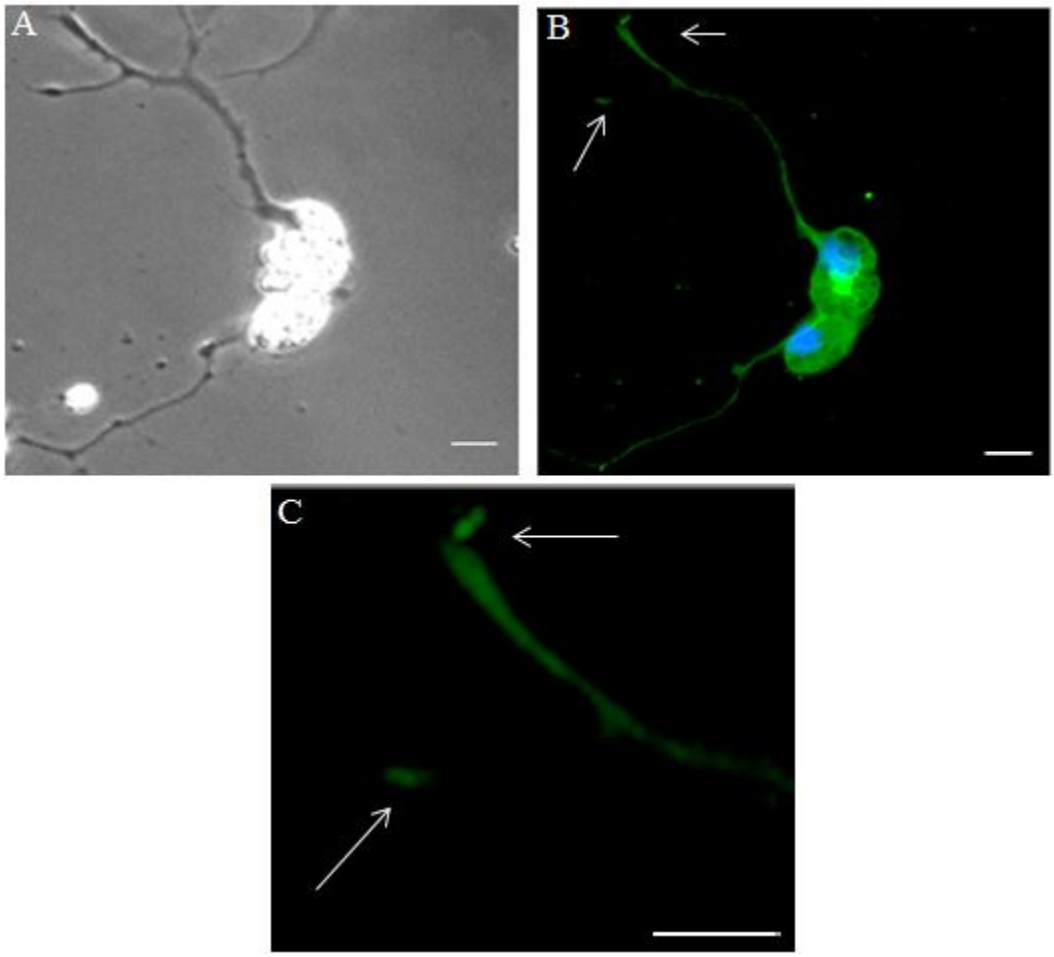

Figure 24. RARβ is present within the cytoplasm, neurite and growth cone of cultured *Xenopus* embryonic spinal cord neurons. A phase contrast image of a fixed spinal cord neuron (**A**) and the immunohistochemical staining for the vertebrate RARβ (green) and DAPI (Nuclear; Blue; **B**) (n = 10). Scale bars = 15 µm. Arrows indicate staining present in the growth cone which is magnified to show more clearly the immunostaining of RARβ within the growth cone (**C**).

Chapter 4: Discussion

In this study, I have provided evidence using cultured motorneurons from the snail, *Lymnaea stagnalis*, that the RXR and the novel, non-chordate RAR, play a role in retinoic acid-induced chemoattraction. In addition, I have shown that both naturally occurring isomers, all-*trans* and 9-*cis* retinoic acid, have a neurotrophic and chemotropic role in vertebrate neurons from the frog, *Xenopus laevis*. Furthermore, I have provided data showing a potential role for the RXR and RARβ in RA-mediated growth cone turning of frog embryonic spinal cord neurons. Using immunocytochemistry, I have also provided data supporting a non-genomic role for RARβ in cultured vertebrate cells whereby immunoreactivity was found in the cytoplasm, neurite and growth cones of embryonic spinal cord neurons.

4.01 RXR mediates 9-*cis* and all-*trans* retinoic acid-induced growth cone guidance

Previous evidence by Farrar (2009) showed that an RXR pan-antagonist, PA452, was unable to block either 9-*cis* or all-*trans* RA-induced growth cone turning, which led the author to speculate from these findings that RXR may not be involved. However, since then Carter *et al*. (2010) showed that PA452, along with another RXR pan-antagonist, HX531, were able to block PA024 (RXR agonist) induced growth cone guidance (with HX531 having more significant inhibitory effects than PA452). In contrast, I have shown that the RXR pan-antagonist, HX531, was able to block guidance of PeA neuron growth cones to both 9-*cis* and all-*trans* RA. Accordingly, Carter *et al*. (2010) indicated that HX531 appeared to have a greater inhibition of RXR pan-agonist-induced growth cone turning as it blocked 9 of 10 PA024-induced growth cone turns whereas PA452 blocked only 3 of 9 growth cone turns. It is therefore possible that PA452 does not have as high affinity for the RXR in *Lymnaea* compared to HX531. Indeed, Takeuchi *et al.* (2010) have suggested previously that PA452 is a selective, but weak RXR antagonist. The recently cloned *Lym*RXR has a high homology with vertebrate RXRα (80%) particularly in the ligand binding domain, which implies that the application of a RXR antagonist designed against the vertebrate RXR, has a high probability of binding and being effective in *Lymnaea* neurons

(Carter *et al*. 2010). Authors have suggested that HX531 is a more effective antagonist of RXR than PA452 (Takeuchi *et al*. 2010) and has previously been shown to block 9-*cis* RA-induced inhibition of HL-60 (Human promyelocytic leukemia cells) cell proliferation and differentiation (Kanayasu-Toyoda *et al*. 2005). It is also of note that the mean turning angles induced by either isomer in the presence of HX531 appears to be more negative than those seen in LE540. It is unknown whether this could be due to the higher involvement of the RXR than the RAR in growth cone guidance, the differing selectivities of the antagonists, or whether RA is now acting as a chemorepellant. In addition it is unknown whether this has any significance to the mechanisms involved in growth cone guidance. The all-*trans* and 9-*cis* retinoic acid-induced growth cone turning angles produced in this thesis were comparable to those previously shown in response to these RA isomers in the presence of DMSO with cultured PeA neurons, indicating consistency between growth cone assays (Farrar, 2009). Therefore, these data strongly support a role for RXR in retinoic acid mediated growth cone turning as being both sufficient and necessary for chemoattraction in *Lymnaea* neurons.

4.02 *Lym*RXR is present in the growth cones which failed to demonstrate a positive turn

Classically, retinoic acid has been known to act through its nuclear receptors, RAR and RXR, however, recently our lab has shown non-nuclear localization of RXR in the cytoplasm, neurites and growth cones of cultured neurons (Carter *et al*. 2010). As previously mentioned, Carter *et al*. (2010) demonstrated that 95 of 102 growth cones examined contained RXR, however some of the neurites showed little or no RXR staining. In order to determine if growth cones which failed to turn toward an RXR agonist, (either PA024 or retinoic acid) in the presence of HX531 was due to a possible absence of the receptor in these particular growth cones, I used immunocytochemistry to illustrate RXR immunoreactivity within these neurites. As anticipated, it was found that in all cases, regardless of whether the growth cone turned toward (i.e. PA024) or away (i.e. all-*trans* RA in HX531) from the retinoid source, RXR was

found to be present in the growth cone. RXR immunoreactivity was also shown for the vertebrate RXRα in the cytoplasm, dendrites and axons of vertebrate neurons (Calderon and Kim, 2007). Taken together, these data further support a non-genomic role for RXR in RA-mediated growth cone turning of *Lymnaea* neurons. In addition to the findings in our lab, there have been previous reports of RXR functioning non-genomically in human platelets (Moraes *et al.* 2007). More specifically, RXR was shown to bind to the G protein, Gq, to inhibit enucleated platelet activation.

Previous work done by Farrar *et al*. (2009) showed that isolated neurites retained the ability to respond to retinoic acid as well as to PA024, indicating that RXR localized in the growth cones of these neurons might be mediating this growth cone turning response. It has been shown, however, that RA can directly bind to and activate other proteins (other than RXR) and thus we cannot entirely rule this out, although evidence supports a role for RXR. For example, RA can activate cAMP response element-binding protein (CREB) independent of RAR and RXR in human tracheobronchial epithelial cells (Aggarwal *et al*. 2006). In fact, inhibition of protein kinase C (PKC) and extracellular regulated kinase (ERK) blocked RA-mediated activation of CREB. In contrast, Farrar *et al*. (2009) showed that inhibition of PKCα using Gö6976 did not significantly block RA-induced chemoattraction suggesting RA-mediated growth cone turning is working through a different mechanism. Taken with my previous data, which showed inhibition of RA-induced chemoattraction by HX531, these data show, at least in *Lymnaea*, that RXR is indeed mediating the response to RA. Final confirmation would require binding studies to definitively show that the agonist and antagonists are binding to RXR in *Lymnaea*.

4.03 All-*trans* and 9-*cis* retinoic acid-induced chemoattraction involves the novel, non-chordate RAR

Here I have shown, for the first time, a role for the novel, non-chordate RAR in RA-induced chemoattraction. Recent data from our lab had demonstrated a role for the recently

cloned *Lym*RAR in embryonic development and due to its localization in the neurites and growth cones of cultured neurons, a role in RA-mediated growth cone guidance was proposed, but not investigated (Carter, 2011). Our lab illustrated non-nuclear *Lym*RAR immunoreactivity patterns similar to that seen with *Lym*RXR in regenerating neurons. In accordance with my hypothesis that the RAR may play a similar role as RXR in RA-induced growth cone guidance, I have shown that application of an RAR pan-antagonist, LE540, significantly reduced the turning angle towards both all-*trans* and 9-*cis* retinoic acid. The idea that RAR plays a role in RA-mediated growth cone guidance is not surprising as previous evidence in our lab showed that the *Lym*RAR shares 100% amino acid identity at the residues thought to interact with RA in the active binding site (Carter, 2011). In addition, the RARβ-selective antagonist, LE135, has been shown to inhibit limb and spinal cord regeneration in the newt and cause embryo malformations in *Lymnaea* (Carter *et al*, 2011; Carter, 2011 Dmetrichuk *et al*. 2005). Taken together, these data strongly support a role for RAR in the developmental and regenerative processes.

It was previously thought that *Lymnaea* did not possess RARs, however questions arose about its presence since its proposed natural ligand, all-*trans* RA, was identified in the *Lymnaea* CNS (Dmetrichuk *et al*. 2008). Because both RA isomers are known to bind RAR in vertebrates, it is not unexpected that LE540 was able to block all-*trans* and 9-*cis* RA-induced growth cone turning. Prior work with the RAR antagonists, LE540 and LE135, had not shown any direct evidence of an interaction between all-*trans* RA and RAR (Carter *et al*. 2011). However, recent work by Campo-Paysaa *et al*. (2008) predicted the binding of all-*trans* RA to the RAR from the mollusc *Lottia gigantea* based on ligand binding domain conservation. These recent data, along with my present results, provide evidence for all-*trans* RA acting through RAR in the mollusc and supports a novel role for RAR in growth cone guidance in cultured invertebrate motorneurons.

4.04 Growth cone guidance through RXR is retained in the presence of an RAR antagonist

Recently, Carter *et al*. (2010) showed that when PA024 was focally applied to regenerating growth cones in the presence of PA452 or HX531, both antagonists blocked RXR agonist-induced growth cone turning. This evidence suggests that the agonist is likely relatively selective for *Lym*RXR and may have little cross-reactivity with RAR, however, this does not show selectivity of the RXR antagonists. Furthermore, we were unsure if the RAR pan-antagonist, LE540, cross-reacts with RXR and that the LE540 inhibition of RA-induced chemoattraction was in part due to that. As stated previously, LE540 has been shown to be much less selective for the vertebrate RARs than LE135. Binding assays revealed binding of LE540, albeit with low affinity, to the vertebrate RXR subtypes, yielding a 3-7 fold lower affinity than for RAR subtypes (Umemiya, 1997).

Interestingly, I showed that PA024 is able to retain its ablilty to attract *Lymnaea* growth cones even in the presence of LE540 (Figure 16; $p>0.05$), suggesting there is little cross-reactivity of LE540 with *Lym*RXR. These data support previous literature that showed that the combination of an RXR agonist (SR11234) with an RAR antagonist (Ro41-5253) is still able to induce differentiation and apotosis of promyelocytic cells (Botling *et al*. 1997). Moreover, activation of RXR has been shown to induce differentiation of stem cells to a muscle lineage, independent of RAR activity (Le May *et al*. 2011). Activation of RXR independent of RAR is best illustrated by my results showing that PA024 is able to induce growth cone turning despite the presence of LE540, presumably blocking RAR activity. Although these data provide evidence that RXR may act independently of RAR to mediate chemotaxis to the RXR agonist, it remains unclear whether this is the case in response to retinoic acid.

It is possible that in the case of RA-induced growth cone guidance, RA binds both RAR and RXR and induces heterodimerization to elicit chemoattraction, so neither receptor can act alone. On the other hand, it is possible that RA first activates RAR which heterodimerizes with

RXR to elicit its effects and therefore, antagonizing either of the receptors renders RA inactive. Heterodimerization of RAR/RXR has mainly been shown in vertebrates, however there is one example in the invertebrate chordate, *Polyandrocarpa misakiensis* (budding ascidian), where RAR and RXR heterodimerize and act as transcriptional activators in the presence of RA (Kamimura *et al.* 2000). In the case of the synthetic RXR ligand, binding only to RXR may allow it to operate as a homodimer independent of RAR or as a heterodimer with potential orphan receptors to elicit growth cone turning. The RXR is known to heterodimrize with several orphan receptors including peroxisome proliferator-activated receptor (PPAR), nerve growth factor- induced clone B (NGFIB) and the nuclear receptor related 1 (Nurr-1) which is located in dopaminergic neurons (Mangelsdorf and Evans, 1995). It has previously been suggested that RXR-selective agonists alone cannot induced RAR/RXR heterodimerization and therefore RA-mediated events (Gronemeyer *et al*. 2004), but it is unclear if this would hold true in invertebrates. Because this is merely speculation, further experiments are required to determine the true course of action with which RA stimulates chemotaxis.

Although the mechanism by which RA elicits its effects remains unclear, evidence provided here shows that effects of two different RA isomers were blocked by HX531 and LE540. There is growing controversy regarding the binding affinities for each isomer to RAR and RXR, however evidence that the RAR pan-antagonist, LE540, is able to block both all-*trans* and 9-*cis* RA-induced chemoattraction, further supports the idea presented in vertebrates that both isomers bind RAR equally (Maden, 2007). Furthermore, data illustrated here showed that the RXR pan-antagonist, HX531, blocked both all-*trans* and 9-*cis* RA-induced growth cone guidance suggesting these two different isomers both bind RXR in *Lymnaea*. This supports previous literature that both isomers bind RXR equally in the locust (Nowickyj *et al*. 2008). Furthermore, experiments in *Drosophila* and mouse showed that all-*trans* RA appeared to have lower affinity for RXR compared to 9-*cis* RA, but when you consider that all-*trans* RA is

thought to be present at much higher concentrations than 9-*cis* RA, its binding to RXR may have physiological relevance (Heyman *et al*. 1992; Ulven *et al*. 2001). Moreover, the evidence in this study that both antagonists are able to block both isomers suggests these two different isomers may be working through the same pathway to elicit their effects.

4.05 Activation of the *Lym*RAR may not be sufficient to induce growth cone turning

Because the RAR pan-antagonist, LE540, showed nearly complete inhibition of RA-induced growth cone turning, I assessed whether the RAR pan-agonist, TTNPB, could induce growth cone turning similar to that seen with not only RA, but also the RXR pan-agonist PA024. When compared to the focal application of DMSO, TTNPB was unable to induce a growth cone turning that was significantly larger than that produced by the vehicle control. This result may suggest that TTNPB does not bind as efficiently with the *Lym*RAR as it has been shown to in vertebrates or that activation of the RAR alone is not sufficient to elicit growth cone guidance. Interestingly, bathing *L. stagnalis* embryos in the RAR pan-agonist, TTNPB, did not show malformations at 10^{-6}M like those seen with either RA or RAR antagonists (Carter, 2011). However, increasing this concentration to 10^{-5}M caused complete lysis of the embryos, similar to that seen in higher concentrations of the antagonists. Furthermore, TTNPB appeared to induce cell death of cultured *Lymnaea* neurons at a concentration of 10^{-5}M (Vesprini, 2011). It may be that TTNPB has a weaker binding affinity for RAR when compared to the natural ligand (i.e. all-*trans* RA), however when it does bind, TTNPB might have more toxic effects. This is consistent with previous reports that TTNPB can be more teratogenic than all-*trans* RA in different vertebrate species (Howard *et al*., 1989; Kistler *et al*., 1990; Kochhar, 1987; Loeliger *et al*., 1980, Pignatello *et al*. 1999). In addition, similar teratogenic effects induced by RA were seen with TTNPB in frog tadpoles (Maden and Corcoran, 1996), indicating TTNPB may be able to mimic some of the effects seen with retinoic acid.

During the growth cone assays, TTNPB was applied at a concentration of 10^{-5}M in the pipette, which could theoretically result in approximately 100-times lower concentration near the growth cone (i.e. 10^{-7}M; Lohof *et al.* 1992). Previous studies showed that TTNPB was able to effectively activate vertebrate RAR at concentrations as low as 10^{-8} M (Pignatello *et al*. 2002). Interestingly, Pignatello *et al*. (1999) found that TTNPB had a 10-fold lower affinity for RARs when compared with all-*trans* RA. This evidence suggests that perhaps a 10-fold higher concentration of TTNPB in the pipette (10^{-4}M or 10^{-6}M at the growth cone) may elicit similar growth cone turning angles seen in response to RA. Therefore, it still remains unclear as to the binding affinity of TTNPB for *Lym*RAR and binding assays would be required to determine if these results are indicative of RARs insufficiency to induce growth cone turning or TTNPBs inability to bind *Lym*RAR.

4.06 Retinoic acid's neurotrophic and chemotropic role in *Xenopus* neurons involves RXR and RARβ

Previous studies have shown a role for several neurotrophic agents in the frog, *Xenopus laevis* including BDNF, NGF and NT-3 (Zheng *et al*. 1994; Song *et al*. 1997, Ming *et al*. 1997, 2001; Song and Poo, 1998). As previously stated, several of these molecules have been shown to direct growth cones of cultured embryonic spinal cord neurons acting as chemoattractive or chemorepulsive agents. Because the role of RA as a chemotropic molecule in an invertebrate has been well documented, I aimed to determine if there is conservation of this role in individual cultured *Xenopus laevis* embryonic spinal cord neurons, as this had yet to be investigated. However, like many investigations before, I first looked at the potential neurotrophic role that RA may play in the frog, as this had not been shown in this species.

For the first time, I showed that both naturally occurring isomers all-*trans* RA and 9-*cis* RA promoted neurite outgrowth from cultured *Xenopus* embryonic spinal cord neurons when compared to cells cultured in the presence of ethanol. In accordance, previous investigations by

Maden *et al.* (1998a) showed all-*trans* RA's ability to induce neurite outgrowth in chick neural tube cells. Interestingly, these researchers found that at the same concentration used in our experiment (10^{-7}M), only neurite length was significantly increased (as opposed to the number of neurites), similar to the findings presented here. It has also been shown that all-*trans* RA in combination with the neurotrophic agents NGF or NT-3, enhances survival and neurite outgrowth of embryonic chick sympathetic neurons (Plum *et al*. 2001). Furthermore, all-*trans* RA was shown to induce significantly more outgrowth of newt spinal cord explants, both in number and length (Dmetrichuk *et al*. 2005). The majority of studies in the literature have focused on the effects of *all-trans* RA on vertebrate regeneration, however there is growing evidence of a role for 9-*cis* RA in limb regeneration (Viviano *et al.* 1995). In addition, earlier work in our lab showed the same neurotrophic effect on newt spinal cord explants with 9-*cis* RA (Rand, 2009; Zeglinski, 2008), coinciding with the effect seen on cultured spinal cord neurons from the frog. Based on these data and evidence that endogenous RA is present throughout development in *Xenopus* and many other amphibians (Maden, 1996), it can be said that retinoic acid may play a significant role in the regenerative process of the vertebrate CNS and that 9-*cis* retinoic acid may play a larger role than previously thought.

Although investigators have shown directed outgrowth of newt spinal cord explants (Dmetrichuk *et al*, 2005) and chick neural tube cells to a gradient of all-*trans* RA (Maden *et al*. 1998), growth cone turning assays had not been performed on single vertebrate growth cones to conclusively show a chemotropic role for all-*trans* RA in vertebrate neurons. In addition, little to no evidence indicating a chemotropic role for 9-*cis* RA in vertebrates has been shown. For the first time, I showed that endogenous RA isomers, all-*trans* and 9-*cis* RA were able to induce significantly higher mean growth cone turning angles compared to focal application of ethanol alone. This is in accordance with previous studies using all-*trans* and 9-*cis* RA soaked beads which were shown to guide newly regenerating newt spinal cord explant neurites toward the

bead (Dmetrichuk *et al*. 2005; Rand, 2009, Zeglinski, 2008). Due to the paucity of studies on RA-induced growth cone turning in vertebrate neurons, little is known regarding the receptor(s) mediating this response. Thus, I aimed to show the potential role of RAR, specifically the β subtype (since previous studies in our lab have implicated this receptor in directed outgrowth from spinal cord) (Carter, 2011; Carter *et al*., 2011; Dmetrichuk et al. 2005), in mediating this turning behavior to its proposed natural ligand, all-*trans* RA. Interestingly, all-*trans* RA-induced growth cone turning was blocked in the presence of the RARβ-selective antagonist, LE135. This is not the first time RARβ has been implicated in a regenerative process as RARβ2 was suggested as the primary mediator of the RA regenerative signal in the mouse DRG and was shown to stimulate neurite regeneration in the mouse spinal cord (Corcoran *et al*., 2000; Corcoran *et al*. 2002). Dmetrichuk *et al*. (2005) also showed that LE135 significantly reduced neurite outgrowth of newt spinal cord explants elicited by all-*trans* RA. Furthermore, LE135 was shown to inhibit newt tail and caudal spinal cord regeneration (Carter *et al*., 2011). Moreover, RARβ2 expression was found to be up-regulated in the regenerating newt tail and the protein is present in the spinal cord during tail regeneration (Carter *et al*., 2011). Taken together, these data strongly support a role for RARβ not only in limb and spinal cord regeneration, but also growth cone guidance by mediating the response to all-*trans* RA. Furthermore, expression of a dominant-negative RARβ in *Xenopus* embryos interfered with retinoid teratogenesis and induced specific hindbrain and neural tube abnormalities (van der Wees *et al*. 1998), suggesting RARβ mediates the developmental effects of RA. Given the evidence for a role for RARβ in CNS regeneration, especially in vertebrates, I believe it would be interesting to test if an RARβ-selective agonist (i.e. CD2019; Agudo *et al*. 2010) would be able to elicit positive growth cone turning as this agonist has been shown to overcome inhibition of axonal outgrowth in the rat spinal cord (Agudo *et al*. 2010). Furthermore, TTNPB, an RAR pan-agonist, which was designed against the vertebrate RARs, may be able to induce similar growth cone turning behavior seen

with RA, even though it failed to do so in the invertebrate preparation. If these agonists were able to induce positive growth cone turning, it would suggest that the vertebrate RAR is sufficient to mediate the effects of retinoids on growth cone turning. However, testing the effects of adding the RXR agonist, HX531, to the bath while applying the RAR agonist would determine whether activation of both RA receptors is required for this morphological response, or whether the receptors may act independently.

As we have shown, in addition to the ability of all-*trans* RA to guide vertebrate growth cones, 9-*cis* RA was able to produce a mean turning angle significantly higher than application of ethanol alone. Subsequently, I decided to assess the role that the RXR may play in mediating this response, as previous literature suggests that 9-*cis* RA is the primary ligand for RXR in vertebrates, however RAR is thought to be the primary receptor for all-*trans* RA. Bath application of the RXR pan-antagonist, HX531, significantly reduced the mean turning angle induced by 9-*cis* RA suggesting a role for this receptor in this turning behavior in vertebrate embryonic neurons. This is the first evidence that RXR may mediate growth cone guidance in a vertebrate model system. Although RXR has not been implicated in growth cone guidance, it has been implicated in several species as playing a part in neural regeneration and protection. Several RXR isoforms were present in the cytoplasm of cultured rat hippocampal neurons (Calderon and Kim, 2007) and RXRα was, interestingly, localized in the axonal compartment of regenerating rat sciatic nerves (Zhelyaznik and Mey, 2006) and dendrites of rat spinal cord tissue (Schrage *et al*. 2006). Moreover, adult mice with a mutated RXRγ show disruption in neuronal plasticity in the hippocampal area, suggesting a possible disruption of growth cone guidance required to form new synaptic connections (Chiang, *et al*. 1998). Furthermore, artificially activating RXR with a synthetic ligand promotes survival of rat dopaminergic neurons *in vitro* (Wallen-Mackenzie *et al*. 2003). In *Xenopus*, by artificially increasing RA levels and more specifically RXRβ, the number of primary neurons in the developing embryo significantly increases, showing that this receptor

is active in the neural plate and tube, the area from which these neurons were isolated (Maden, 2007). Based on the evidence presented here, it can be said that RXR plays a role in not only neuroprotection, but neuronal regeneration and that it may mediate retinoic acid-induced chemoattraction. It would therefore be interesting to test whether the RXR pan-agonist, PA024, would be able to induce positive growth cone turning, similar to that seen in *Lymnaea*, and therefore whether the RXR is capable of acting independent of RAR, as this has not been shown in this species.

4.07 Non-nuclear localization of RARβ in cultured embryonic spinal cord neurons

Based on tissue immunohistochemistry in the newt tail and spinal cord regenerate, it had been suggested that RARβ may act outside the nucleus (Carter, 2011). However, non-nuclear staining of a vertebrate RARβ has not been shown in individual vertebrate neurons, although it has recently been illustrated in the invertebrate model. Subsequently, I employed immunocytochemistry to determine the distribution of RARβ in cultured embryonic *Xenopus* spinal cord neurons. Interestingly, I showed that in all 10 neurons stained for the vertebrate RARβ, all showed non-nuclear immunoreactivity, where RARβ was localized in the cytoplasm, neurites and growth cones. This is a pattern similar to that seen previously with RAR and RXR in *Lymnaea* motorneurons (Carter, 2011). The antibody used in this experiment was a custom-made antibody against a synthetic peptide from the hinge region between the DNA-binding domain (DBD) and the ligand binding domain (LBD) of the newt, *Notopthalmus viridescens*, RARβ2. The *Nv*RARβ2 shares ~90% amino acid identity with the *Xenopus* RARβ. In fact, the custom made antibody epitope aligns in a highly conserved amino acid sequence between the newt and *Xenopus* RARβ (Figure 25), which indicates that the immunofluorescence observed in cultured *Xenopus* spinal cord neurons can likely be attributed to RARβ . These data show for the first time, non-nuclear staining of a vertebrate RARβ in single vertebrate neurons and provides strong evidence supporting a non-genomic role for this receptor in neurite outgrowth and RA

mediated chemoattraction. This is not the first evidence showing non-nuclear localization of RAR in vertebrate cells and tissues as RARα and β were shown to be present in the cytoplasm of rat Schwann cells following sciatic nerve injury (Zhelyaznik and Mey, 2006). In addition, *Nv*RARβ2 was shown to be localized in ependymal cells surrounding the central canal of the spinal cord, the outer perimeter of spinal cord (meninges) and cytoplasm of cells in the wound epidermis, all in the regenerating newt tail (Carter *et al*., 2011). Evidence presented here and previously, supports the hypothesis of a non-genomic role for RARβ in spinal cord and limb regeneration as well as RA-mediated chemoattraction.

This would not be the first time a non-genomic role for RAR has been suggested however, in fact there are several known examples of vertebrate RARs functioning non-genomically. Liao *et al*. (2004) demonstrated that RARβ-selective agonists could elicit neurotransmitter release from the *Xenopus* neuromuscular junction independent of the cell body. These data are intriguing since the cells used in my own *Xenopus* experiments were cultured from the neural tube and surrounding embryonic tissue containing a heterogeneous mix of neuronal and non-neuronal cells which have been shown to form functional neuromuscular junctions in culture (Chow and Poo, 1985). Furthermore, cytoplasmically localized RARs were shown to activate both Erk (1/2) and subsequently CREB when bound by retinoic acid in rat cerebrocortical and DRG neurons (Canon et al. 2004). Moreover, translational repression of dendritically localized GluR1 mRNA by RARα has been demonstrated and this repression was lifted when bound by retinoic acid (Poon and Chen, 2008). Although my data suggest, but do not definitively show a potential non-genomic role for RARβ in RA-mediated chemoattraction and neurite outgrowth, we would need to repeat prior experiments by Farrar *et al*. (2009) using isolated growth cones as well as using local transcriptional inhibitors (i.e. actinomycin D) to determine if RARβ is indeed working through a non-genomic mechanism. This technique may be more difficult with *Xenopus* spinal cord cell cultures, as neurite extensions are not as long or

robust as those seen in *Lymnaea* and there is no evidence indicating whether *Xenopus* neurites survive without the cell body or for how long.

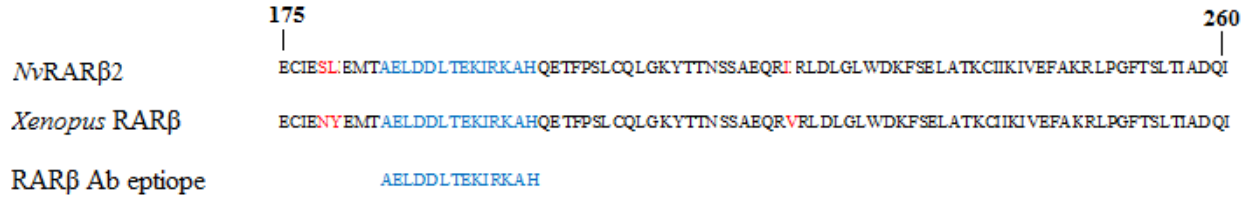

Figure 25. Protein sequence alignment. Basic local alignment search tool (BLAST) alignment of the newt RARβ-2 sequence against the *Xenopus* RARβ and the custom made RARβ antibody (Ab) epitope. Alignment shows the conserved sequence where the antibody binds each protein indicating the fluorescence seen in immunohistochemistry can be attributed to RARβ (National Centre for Biological Information; NCBI).

4.08 What pathway is involved in the growth cone in response to RA application?

i. Orphan Receptors

This work, for the first time, shows evidence for the conservation of the neurotrophic and chemotropic role of retinoic acid in the frog, *Xenopus laevis*. In addition, this work demonstrates the chemoattractive properties of RA at the level of individual vertebrate growth cones for the first time. Although the mechanism involved in this process is still unknown, several possibilities exist. As alluded to earlier, based on results from work with the RAR and RXR antagonists, a potential main player involved in mediating the response to both isomers of RA may be the orphan receptors with which RXR binds, the first of which is Nurr-1. In Nurr-$1^{-/-}$ rats, dopaminergic neurons fail to develop. The authors suggested that perhaps activation of RXR and therefore Nurr-1 via RA, may promote the development and subsequently the regeneration of these cells (Zetterstrom *et al*., 1997). Although Nurr-1 is present only in vertebrates, there is an invertebrate ortholog of the same nuclear receptor subfamily 4 (NR4). This receptor was originally identified in *Drosophila*, NR4A4, also known as *Drosophila* hormone receptor-like 38 (DHR38; Wu and LoVerde, 2008). DHR38 is active throughout development in *Drosophila*, and

much like Nurr-1 in vertebrates, acts to induce differentiation of neuronal tissues to a dopaminergic fate (Davis *et al*. 2007). Another potential receptor involved may be PPAR which is located in peroxisome membranes throughout the cytoplasm and was first identified in *Xenopus* (Dreyer *et al*. 1992). The PPAR/RXR heterodimer has been shown to be formed and able to induce gene expression in response to 9-*cis* retinoic acid (Kliewer *et al*. 1992). Furthermore, non-genomic effects of PPARγ have been shown in which activation of this receptor in enucleated platelets resulted in inhibition of aggregation and calcium mobilization (Moraes *et al*. 2009). Although PPARs have only been identified in vertebrates, there is evidence of a closely related orphan receptor, E75, identified in *Drosophila* (Schoonjans *et al*. 1996). Taken together, these data elucidate a potential role for Nurr-1 or PPAR in mediating RA-induced growth cone turning in conjunction with RXR. In order to elucidate the role these receptors might be playing, it would be worthwhile to conduct growth cone assays in the presence of commercially available Nurr-1 or PPAR antagonists along with the RAR pan-antagonist, LE540 while focally applying the RXR pan-agonist. This would determine if RXR-mediated growth cone turning is acting through a homodimer or perhaps through these orphan receptors.

ii. Calcium

In addition to working through its receptors, RAR and RXR, there is a vast amount of literature on RA's ability to modulate cell responses through different intracellular mechanisms, one of which is calcium. Previous evidence has shown that RA exposure can increase intracellular calcium levels in human (Gao *et al*., 1998; Launay *et al*., 2003) and rat (Short *et al*. 1991) cell lines. In addition, RA has been shown to upregulate calcium binding proteins, thus altering a cell's repsonse to calcium signals (Lawson *et al*. 1999; Chen and Napoli, 2008). Furthermore, intracellular calcium levels are known to be highly involved in the promotion of neural regeneration following injury and calcium flux plays a crucial role in neurite extension

and growth cone behaviour *(*Henley and Poo, 2004; Ibarretxe *et al*., 2007; McClellan *et al*., 2008; Song and Poo, 1999*)*, a phenomena RA is known to induce. Recent evidence from our lab has shown that application of RA to cultured Visceral F neurons from *Lymnaea* causes a significant reduction in intracellular calcium levels. This calcium decrease was, however, shown only at high levels of RA, (contrary to previous evidence of the ablilty of RA to increase calcium levels), although this work was done in adult neurons (Vesprini, 2011) as opposed to immature, differentiating neurons. In relation to growth cone motility and guidance, it has been suggested by several groups that local fluctuations in intracellular calcium are directly responsible for the attractiveness or repulsiveness of various guidance cues. In fact, calcium imaging shows that small elevations in calcium in the growth cone lead to growth cone repulsion, whereas medium spikes in local calcium results in attraction of the growth cone (Henley and Poo, 2004; Wen *et al*. 2004). Interestingly, large elevations in local calcium levels induce repulsion of growth cones. (Robles *et al*. 2003). Moreover, evidence by Farrar (2009) showed that application of the known calcium channel blocker, cadmium, to *Lymnaea* neurons, caused inhibition of all-*trans* RA induced growth cone turning, suggesting that influx of calcium is important in this response. Finally, Song and Poo (1998) showed that *Xenopus* spinal cord neurons cultured in low calcium saline lost their attractiveness to the chemoattractant guidance cues BDNF and NGF. Though this evidence strongly supports a role for calcium in regeneration and more specifically, RA-mediated regeneration and chemoattraction, investigations showing RA's direct effect on calcium levels at the growth cone level have yet to be performed.

iii. cAMP and PKA

Substantial work has indicated the interaction between RA signaling and other intracellular signaling cascades, most notably the cAMP pathway, which is regulated by the enzyme, protein kinase A (PKA). RA has been shown to activate the cAMP response element-binding protein (CREB) independent of its receptors, RAR and RXR (Aggarwal *et al*. 2006).

This mechanism is unlikely in our case as work with RAR and RXR antagonists would not have been successful in blocking RA-mediated growth cone turning. However, direct interactions between RA and PKA have been well documented, for example, PKA inhibition negates RA-induced cell survival (Kholodenko *et al*., 2007) and RARβ-2-induced neurite outgrowth (Wong *et al*. 2006). Moreover, RA-induced increases in RAR mediated transcriptional activity have been shown to be inhibited in the presence of a PKA antagonist (Saito *et al*. 2010). Not only does PKA appear to mediate RA's neurotrophic effects, it has been shown to mediate growth cone turning in response to other tropic factors. More specifically, inhibition of PKA in cultured *Xenopus* embryonic spinal cord neurons caused previously attractive guidance cues to become repellant, much like experiments done in low calcium. Interestingly, activation of PKA caused previously repellant guidance cues to become attractive (Song and Poo, 1998). This evidence shows that high levels of cAMP result in chemoattraction and conversely, low levels lead to chemorepulsion. Therefore, it is entirely possible that RA regulates cAMP levels through PKA. It may act as an agonist either directly or indirectly through the RAR or RXR to raise levels within the growth cone resulting in an attractive response by the growth cone. Although, direct evidence for the role of PKA in RA-mediated growth cone turning has not yet been shown, it would be intriguing to test if PKA inhibition resulted in RA becoming a chemorepellant. Evidence in our lab has shown that PKA inhibition did not alter RA-induced electrophysiological effects (Vesprini, 2011), but these may require completely different mechanisms than those utilized in a morphological response.

4.09 RA as a therapeutic agent?

As is evident in the literature, RA has a quite complex and extensive role in several processes from development to regeneration, however the fundamental pathway(s) through which RA elicits its effects must first be characterized before therapeutic use can be considered. However, it appears to have great potential for use in neurodegenerative disease. It has been

shown extensively in invertebrates and vertebrates that RA has the capacity to induce significant neural regeneration *in vitro* (Dmetrichuk *et al*. 2005; Dmetrichuk *et al.* 2006) as well as guide axonal processes (Dmetrichuk *et al*. 2006; Dmetrichuk *et al*. 2008; Farrar *et al*. 2009; Carter *et al*. 2010). Furthermore, functional recovery has been shown in rat and mouse models of spinal cord injury and Parkinson's disease whereby pharmacological application of RA or up-regulation of its receptors induced regeneration of neural tissues (Agudo *et al*. 2010; Ulusoy *et al*. 2011; Wong *et al*. 2006; Yip *et al*. 2006). Moreover, faulty RA signaling has been implicated in a number of neurodegenerative diseases, most notably Parkinson's disease (Krezel *et al*. 1998) and motorneuron disease (i.e. amyotrophic lateral sclerosis (ALS); Corcoran *et al*. 2002b). In addition, RA has been thought to play a role in affective disorders like depression (Bremner and McCaffery, 2008) and schizophrenia (Krezel *et al*. 1998). Although evidence supporting the use of RA as a therapeutic agent is growing, potential side effects and interactions with other pathways have yet to be identified before RA's potential benefits can be applied.

4.10 RXR and RAR antagonist selectivity

The majority of the work presented here relied upon the presumed selectivity of synthetic antagonists of the retinoid receptors, RXR and RAR. Since these molecules were designed against vertebrate receptors, it is unknown how selective they are for the *Lymnaea* receptors and therefore any data presented can only suggest, but not definitively show, a role for either of the retinoid receptors. However, *Lym*RXR has a relatively high overall homology to rat and human RXR (~80%) and 100% amino acid identity at the specific residues thought to interact with RA (Carter *et al*. 2010). Therefore, data showing uninhibited growth cone turning in response to the RXR pan-agonist, PA024, in the presence of the RAR pan-antagonist, LE540, suggest relatively high selectivity for the RXR. However, growth cone assays performed using embryonic spinal cord neurons from *Xenopus* can be more easily relied upon. The reason for this is due to the high homology between *Xenopus* RARβ and RXR compared to other vertebrates like the rat and

human where the amino acid identity is ~90% between these receptors. As stated previously, binding assays for these synthetic agonists and antagonists are required in order to conclusively show the role of the retinoid receptors in RA-induced growth cone turning.

4.11 Summary

In this study, I have shown that RA-induced chemoattraction of *Lymnaea stagnalis* PeA motorneuron growth cones is mediated by both the RXR and RAR. In addition, RXR-mediated growth cone turning, at least in response to activation by the synthetic agonist, appears to act independently of the RAR, potentially through parallel pathways which have yet to be identified. For the first time I showed that RA's neurotrophic and chemotropic role in invertebrates is conserved in frog embryonic spinal cord neurons, where both isomers of RA, all-*trans* and 9-*cis*, induced significant neurite outgrowth as well as positive growth cone turning. Furthermore, all-*trans* RA-induced chemoattraction may be mediated by RARβ, whereas 9-*cis* RA-induced growth cone guidance may act through RXR. Interestingly, RARβ was localized in a non-nuclear distribution in cultured embryonic spinal cord neurons, potentially indicating a novel, non-genomic role for RARβ in vertebrate neurite outgrowth and chemoattraction. These results provide further evidence supporting a conservation of the retinoid signaling pathway in vertebrate neurons and indicate that the same receptors may be mediating the chemoattractive response induced by RA.

Chapter 5:

Appendix 1

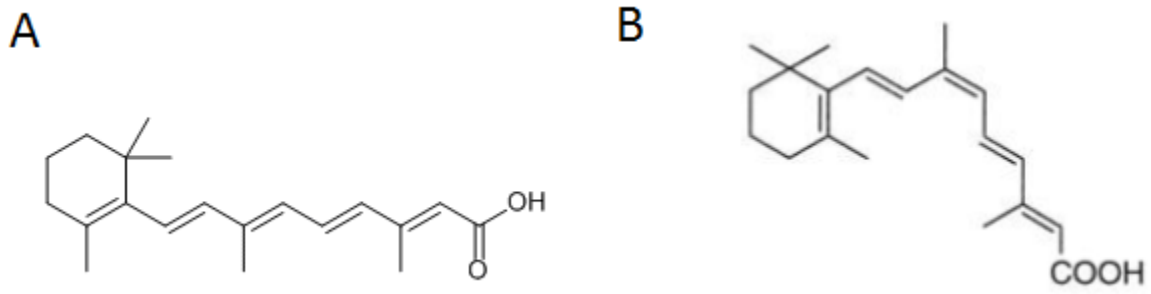

Figure 26. **Two isomers of retinoic acid. A.** all-*trans* retinoic acid. **B.** 9-*cis* retinoic acid.

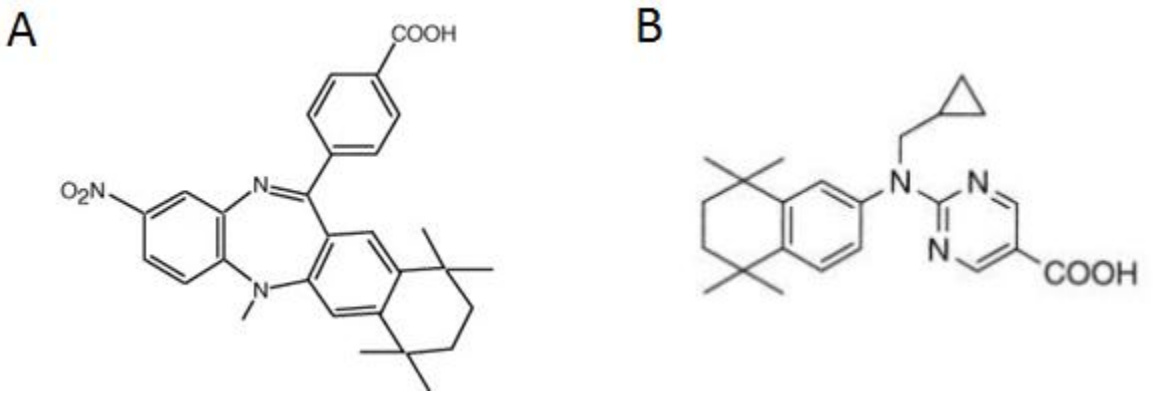

Figure 27. Structure of the synthetic RXR ligands. A. RXR pan-antagonist HX531. **B.** RXR pan-agonist PA024.

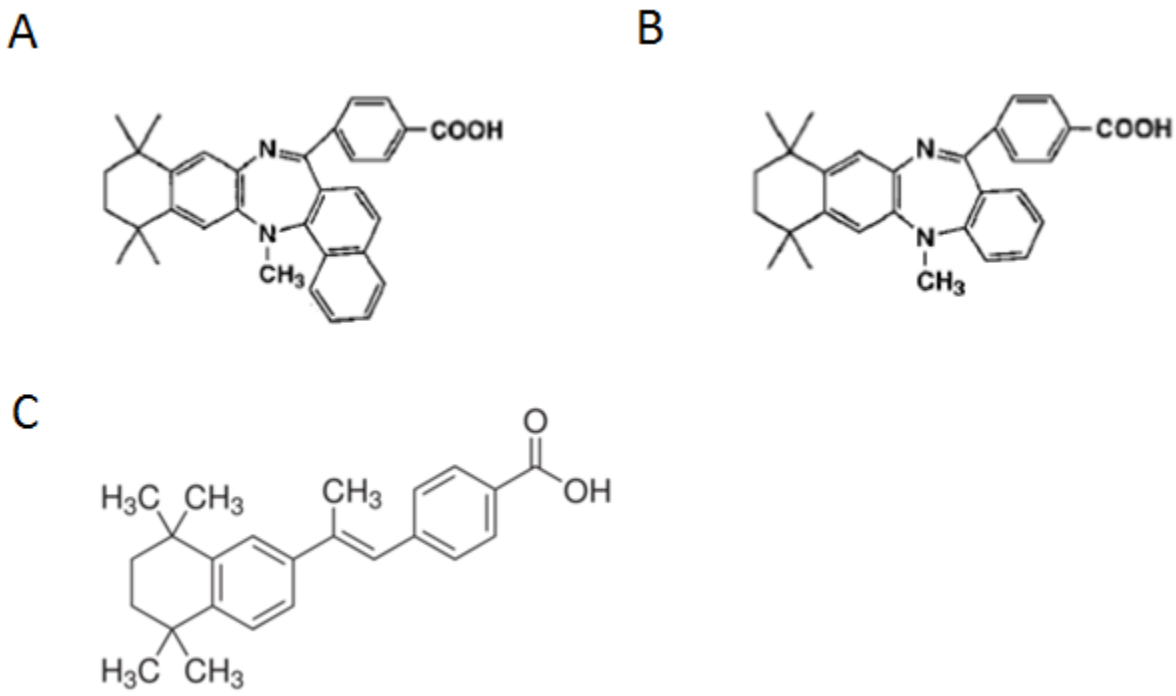

Figure 28. Structure of the synthetic RAR ligands. A. RAR pan-antagonist LE540. **B.** RARβ-2 selective antagonist LE135. **C.** RAR pan-agonist TTNPB.

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